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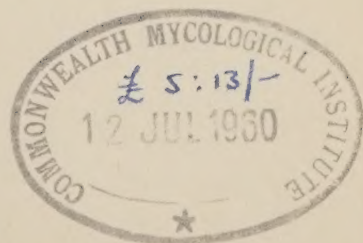
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Chairman of the Symposium

HENRY WELCH, Ph.D.

Under the Editorial Direction of

FELIX MARTI-IBÁÑEZ, M.D.

ANTIBIOTICA, INC.

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Dr. Henry Welch (*left*), chairman of the Symposium, delivering the opening address. Dr. Félix Martí-Ibáñez (*right*), who spoke on "The Great Historical Challenges in Medicine."



The opening session of the Seventh Annual Symposium on Antibiotics, held November 4 to 6 in Washington, D. C.



Dr. Austin Smith (*left*), who spoke on "The Challenge of New Drugs to the Pharmaceutical Industry." Dr. Maxwell Finland (*right*), who spoke on "The Challenge of New Drugs to the Clinical Investigator."



Dr. William H. Kessenich (*left*), who spoke on "The Challenge of New Drugs to the Food and Drug Administration." Dr. John J. Curry (*right*), who addressed the opening session on "The Challenge of New Drugs to the Practicing Physician."

THE WHITE HOUSE

WASHINGTON

November 2, 1959

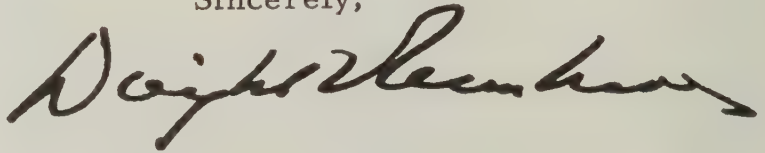
Dear Dr. Welch:

Please give my greetings to those participating in the Seventh Annual Symposium on Antibiotics.

It is gratifying to learn of the distinguished group of scientists from many lands who have come to Washington on this occasion to discuss the latest findings of research in the field of antibiotics. This Symposium is an example of international cooperation at its best, and I am sure it will bring us closer to the peaceful and productive world which is the goal of mankind.

I am delighted to add my best wishes for a most successful Symposium.

Sincerely,

A handwritten signature in dark ink, appearing to read "Dwight D. Eisenhower". The signature is fluid and cursive, with a long, sweeping underline that extends to the right.

Dr. Henry Welch
Chairman
Seventh Annual Symposium on Antibiotics
Mayflower Hotel
Washington, D. C.

Opening Remarks

HENRY WELCH

There has been a steady and progressive increase of interest in this Antibiotic Symposium during the past seven years, not only from those clinicians and investigators in the United States working in this field but also from our colleagues abroad. This year nearly 300 abstracts of papers were submitted to the program committee for review and of these 79 were received from 22 foreign countries, including Argentina, Austria, Brazil, Canada, Czechoslovakia, Chile, Ecuador, England, France, Germany, Haiti, Italy, Japan, Mexico, Panama, Peru, Poland, Portugal, Scotland, South Africa, Sweden, and Uruguay. Representatives from all but two of these countries will deliver papers during the three day sessions.

As in the past, we have planned an opening session of general interest entitled "The Challenge of New Drugs in Our Society." The tremendous sums of money made available for research from both industry and government sources has resulted in a veritable deluge of new chemotherapeutic agents. This enormous number of new drugs reflects the energy and effort now being directed by our pharmaceutical industry to research and development, as contrasted to the historical beginnings of Ehrlich and his search for the "magic bullet" that would cure syphilis. This challenge of new drugs is perhaps the most difficult challenge medicine has encountered in its long and arduous history.

From the 278 abstracts of papers reviewed by the program committee, it was necessary to limit to only about 150 the papers selected for presentation. It was a difficult task, but I believe the Committee has done as good a job as possible with the mass of material they had to consider. They regret that because of time limitations it was necessary to have a large number read by title and to reject others, mostly because they duplicated previously presented material. The committee wants to be sure it is understood by all authors that each paper on the program, whether to be read or listed by title, was considered of equal merit. Those chosen for presentation were the ones the committee felt would give a well rounded program.

Again this year two panel sessions will be held, one moderated by Dr. Criepp, on the problems that confront us in human sensitization to antibiotics, and the other, moderated jointly by Doctors Ross and Sprunt, on the treatment of acute and chronic infections in pediatrics and geriatrics. I think you will find both panels interesting and informative. Four sessions were arranged by the program committee covering, respectively, Pediatrics, Venereal Disease, Dermatology, and Methodology for Determining the Susceptibility of Microorganisms. This program arrangement is an innovation and it is hoped it will prove more acceptable because of its practical usefulness than some of our previous ones which were arranged, usually, on an individual drug basis.

Among the new antibiotics to be reported this year are colistin, aspartocin, ferulenin, streptozotocin, and rifomycin. Of these, colistin, a Japanese discovery, appears to stand out as the one sufficiently studied and ready for use in this country. Of the others, rifomycin is reported to have low activity but also very low toxicity and therefore a favorable therapeutic index.

Of the previously reported agents, new uses will be described for paromomycin, amphotericin, and griseofulvin. Studies on paromomycin indicate its usefulness in amebic dysentery and we will hear more of the value of amphotericin in the treatment of deep systemic fungal infections. However, the outstanding antifungal agent appears to be griseofulvin on which a series of papers will be presented describing the area in which this drug will find wide use in dermatology.

This year more will be heard of antitumor agents as two new ones, streptonigrin and diazomycin are added to the list. The former looks more promising than have others reported in the past. Although papers on clinical studies are not scheduled, such studies are in process and it is hoped that early data will be forthcoming in discussion. Further studies with actinobolin, streptovitamin, and a nonantibiotic agent, cyclophosphamide, will be described as will extensive clinical trials with mitomycin C in Japan, all of which should be of interest in this exciting field.

Important contributions to the tetracycline group of antibiotics, namely, the two new compounds, demethylchlortetracycline and pyrrolidinomethyl tetracycline will be reported on Thursday morning. From the evidence it appears that demethylchlortetracycline will accomplish what the present tetracyclines do in susceptible diseases but with a markedly decreased dose. Pyrrolidinomethyl tetracycline by injection is shown to be superior to the presently available tetracycline preparations, since it appears to cause considerably less pain and tissue damage.

In closing, it is my personal opinion that one of the outstanding contributions this year is covered in the reports on the new synthetic penicillins, particularly from the standpoint of their possibilities for the future. To John C. Sheehan in this country, who first synthesized penicillin V and who first converted penicillin G to 6-aminopenicillanic acid and to the English workers, Doyle and Rolinson, who uncovered an economic source of this compound in fermentation broths goes the credit for this unique and important development. It is apparent now that modification of the side chain attached to penicillanic acid may materially affect its antibacterial activity. The cue to this phenomenon is apparent in retrospect from the activity of synnematin, a penicillin with alpha-amino adipic acid as the side chain, described years ago and found quite active against gram-negative organisms, in contrast to the widely used penicillin G, with a benzyl substituent group, active mainly against gram-positive organisms. Innumerable synthetic penicillins, of course, are possible. In this country more than 500 have already been prepared by the Bristol Laboratories. Perhaps here we have a key that in the near future may "open the door" to a broad-spectrum penicillin, to "antifungal penicillins," "antiviral penicillins," or even to penicillins active against tumor cells by the correct manipulation of the "guiding side chain." Since a great many compounds are possible, it becomes a question of guiding development of them in the proper direction. Already preliminary evidence is available concerning the kind of side chain or direction one must pursue to obtain increased stability and greater absorption. Here too, in retrospect, a lead was given by nature through the stable, rapidly absorbed phenoxymethyl penicillin (penicillin V). The new α -phenoxyethyl penicillin, from the data to be presented this afternoon, is far better absorbed by man than is penicillin G. Furthermore, it is better absorbed than penicillin V, from which it differs only by one additional methyl (CH_3) group. It is of interest that the *addition* of a CH_3 group to penicillin V increased its ability to be absorbed by man while the *deletion* of a similar group from chlor-

tetracycline accomplished a similar improvement in absorbability. (Parenthetically, it is unlikely that here there was an “industry trading” of CH_3 groups.)

The future for the synthetic penicillins offers much promise but, as there obviously are literally enormous numbers of such penicillins possible, there similarly is an enormous amount of work to be done to establish the guide lines needed to direct these drugs against specific targets through manipulation of their side chains.

Now I should like to present the moderator of the first session on “The Challenge of New Drugs to Our Society,” Dr. Félix Martí-Ibáñez, who is the dynamic Editor-in-Chief of the Medical Newsmagazine, *MD*. He is also a leading medical historian, noted psychiatrist, and author. It is my honor to introduce Dr. Martí-Ibáñez, who will enlighten us on the seven great medical challenges of the past with his address on “The Great Historical Challenges in Medicine.”

The Great Historical Challenges in Medicine

FELIX MARTI-IBÁÑEZ

Professor and Chairman of the Department of the History of Medicine, New York Medical College, Flower and Fifth Avenue Hospitals; Editor-in-Chief of MD, the Medical Newsmagazine, New York, N. Y.

Once again, we, physicians, investigators, technicians of the pharmaceutical industry, and even a medical historian temporarily a fugitive from his tasks, are assembled on the occasion of the Seventh Annual Symposium on Antibiotics.

For seven years—seven, the magic number that since even before Pythagoras has represented through the ages the ultimate perfection of man's knowledge of numbers and of the heavens—we have made this symposium an international loud-speaker resounding with the preoccupation and the occupation, with the thought and the word, of the standard-bearers of antibiotic medicine, under the direction of Dr. Henry Welch, of whose dynamic scientific personality and tireless labor these meetings have been a shining reflection.

This year I would like to talk to you about the great challenges to Medicine in the past and the men who faced them.

CHALLENGE AND REPLY IN MEDICINE

What counts in the life of men and of nations is not so much their achievements as the challenges that cross their path and particularly the way they face them. In this sense, the history of our seven symposia is the history of the great challenges encountered by antibiotic medicine and of the way scientific investigators have faced them.

At the first great symposium of all, held more than two thousand years ago at the house of Agathon in Athens, by the light of torches that sparked the wine goblets into a myriad of rubies, Eryximachus the physician defined medicine to the other guests, among whom was Socrates, as "the study of the love affairs of the organs of the body." In like manner, we might say that at these annual symposia we have listened to the love affairs between antibiotics and germs, with the latter behaving like frivolous girls, now being vulnerable, now resisting the antibiotic onslaught.

At first sight, these symposia may appear to summarize every autumn the rich scientific harvest of the previous year. But I would say it is the other way round. Getting together here, interchanging impressions, inquiring into one another's work, dreaming together, sharing our hopes—this to a large degree is what determines the harvest of discoveries to be reaped the following year. More than an account of what you have done in the past year, this symposium each year is like the hoisting and unfurling of an inspiring banner that galvanizes you scientists to further action, to do still greater things in the year to come.

It is fitting to inquire if the new scientific challenges uncovered at each symposium are just another facet of these our times, which, like a rose with its thorns, are so full of promise and yet bristle with problems. History tells us that medicine obeys the law of action and reaction, wherein each new scientific step, each medical discovery, starts a chain reaction of challenges that the physician must resolve if he wants to ensure continuity in the history of medicine.

In mythology each new victory of Hercules during his Labors, each new exploit of Ulysses during his Odyssey across the azure waters of the Mediterranean, led only to new perils that the hero had to overcome if he wanted to reach the haven of his dreams safe, sound, and victorious. In science, each conquest engenders new menaces to itself, and the scientist must stand ready to overcome these menaces, if his progress is not to be deterred by setbacks, like the tortoise in the fable.

Thus, science, like human life, is an operation that moves forward. Like life, science also must be fiercely faithful to itself and to its vital authenticity. It must be faithful to the duty of facing its repertory of problems. In life, as in science, one cannot shelve, shirk, or evade problems. They must be confronted with a serene face and a valiant soul, as problems are wont to flee and vanish before the bold tread of the fearless fighter, just as phantoms in a haunted castle vanish before the first glow of dawn that sheds light and life upon everything.

THE SEVEN GREAT CHALLENGES IN THE HISTORY OF MEDICINE

The emergence of challenges on the road of contemporary medicine is nothing new. At every period in history the physician has been beset by tremendous problems, which he has resolved by dint of his genius and ingenuity. We could reduce to seven these great historical challenges to medicine, just as we could reduce to seven contemporary medicine's responses to such challenges, and again to seven—always the magic number—the spiritual virtues of the scientific investigator, on whose ability to respond to such challenges medical progress depends.

The first great challenge arose in the world that emerged from the neolithic world of the flint. "River civilizations" sprang up on sun-blazed steppes, and brown-skinned peoples, originally from Asia, condemned to annihilation by the scorching sun and whirling sands, struggled desperately to survive in the wildernesses. Such were archaic Egypt and Mesopotamia when man answered the challenge of the shapeless world around him, peopled by invisible presences and demons, with the sheltering geometric architecture of pyramids and ziggurats. That was also the time when medicine men tried to master the invisible forces of nature and disease by means of the first pseudoscience, magic medicine. The archaic medicine man resorted to the stars, the livers of sacrificed animals, and auguries to safeguard the life of his people against the demons that caused the spiritual possessions and fevers we now call psychoses and infections respectively.

The second great challenge came on the golden Greek peninsula and its sun-blistered lemon-and-olive groved islands, where the first great philosophies and religions of the western world were born. There, more than 2500 years ago, man for the first time in history dared to face the intellectual challenge posed by the nature of the universe, of man, and of disease. The Greek philosophers, many of them physicians, were the first to use the magnificent instrument of thought to

awaken man's conscience and dignity, and to formulate the concept of disease as a natural process that could be healed by empirical and rational means.

The third great challenge was sheltered by the gloomy skies of the Middle Ages, when the Black Death and other great pandemics threatened to destroy the human race. The men who met this challenge were physician-priests, shut away in the only places where medicine could still be practiced, the monasteries, which, stationed along the great routes travelled by pilgrims, crusaders, and merchants alike, were a dynamic combination of news agencies and centers of bookish research. Theirs was strictly a "book medicine"; nevertheless, in their fight against epidemics and other manifestations of nature's destructive forces they created hospitals, universities, and public health measures, which, together with the Gothic cathedrals and the *Divine Comedy*, were the great medieval contributions to civilization.

The fourth challenge arose in the Renaissance, a period throbbing with the parallel urges to explore the human body and the horizon stretching beyond the seas, both of which until then were *terra incognita*. The fever of the navigators to discover the lands asleep beneath the virgin stars of the western sky was matched by the fever of Renaissance surgeons and anatomists to discover with their scalpels the mysteries hidden beneath the human skin. The challenge posed by the mysterious structure of the organic fabric was answered by hands urged on by the bold and rational thought of the Renaissance surgeons and anatomists.

The fifth challenge was to decipher the secret of the physiology of the human body and its mysterious functions. This problem was unraveled in the Baroque period, when the motion and emotion characteristic of Baroque art were reflected in the concepts applied to scientific investigation, as in the discovery of the circulation of the blood, which was to make *anatomia animata* in space, just as Baroque embryology made *anatomia animata* in time. When dynamic physiology replaced the static anatomy of the past, the way was opened for a series of discoveries in circulatory, digestive, and nervous physiology, on which modern medical science is founded.

The sixth challenge was presented by disease therapy, which at the end of last century was still as empirical as it was two thousand years ago. Clinical and laboratory investigators answered this challenge with immunobiologic, endocrine, chemical and, more recently, antibiotic therapies, calling for a naturalistic and experimental criterion in the treatment of diseases.

The challenge of our own time is to decipher the nature and the biochemical substrate of disease and its natural history, the biological cycles of the pathogenic agents, the enigmas of genetics, and the secrets of ecology, and thus be able to anticipate the biological destiny of man and to make of therapeutics a subtle diagnostic key with which to unlock the last door opening to the threshold of life.

THE CONCEPT OF BIOCHEMICAL RESPONSIBILITY

We have progressed therefore from the primitive *anatomical* notion of disease, which placed all responsibility on one organ only, to the *physiological* notion, which accepted a multiple organ functional responsibility, and from there to the *biochemical* notion, which, ignoring the primitive concept of the diseased organ and

the later concept of multiple alteration, concentrates instead on biochemical responsibility.

This means accepting the *totality* of the pathological disorder, which, though initially it springs from the alteration of one organ, is not limited to the boundaries of that organ, but adheres to the concept that the seat of every disease, however limited it may seem, is the *whole* body, and the disorder is therefore a *general* disturbance of all the organic humors.

We have thus returned to the original humoral notion propounded more than two thousand years ago by Hippocrates, which was later displaced to some degree by Galen's partly anatomical concepts. This is one of the paradoxes in the history of medicine. It took many centuries to agree that diseases did not float mysteriously within the body but were localized in what was successively described in the course of history as organ, texture, fiber, web, membrane, tissue, and cell.

In contrast, we are nowadays reverting to the *humoral* idea of disease, but with the difference—on which rests the progress of our knowledge—that we now agree that diseases, including psychiatric disorders, are localized in organs but affect the totality of the organism due to the efficient correlation mechanisms of the body, particularly the endocrine and neurovegetative systems, and that mysterious, pallid tissular jungle known as the diencephalon and the hypothalamus-pituitary-adrenal axis. This jungle is in our century the site of enthralling explorations which, with the aid of biochemical tools, are destined to make discoveries in the inner space of man more extraordinary than those that rocket ships may make in outer space.

MEDICINE'S SEVEN ANSWERS TO THE CHALLENGE OF OUR TIME

The challenge therefore that Medicine faces today is to protect health and prevent disease by increasing its knowledge of the processes of health and disease.

In facing this challenge, modern medicine has established the basic problems it must first solve, as follows: First, to know the biochemical substrate of health and its alterations, including that of mental processes. This knowledge, which already enables us to look into the chemistry of joy and sorrow and of the genius and the artist, will provide a dynamic tool with which to approach the promotion of health, not from afar, as in the past, but from the innermost redoubts of its formerly impregnable citadels. Second, to know thoroughly the processes of growth, convalescence, aging, and death, so as to be able to rehabilitate the diseased, to safeguard the development of the human being, and to prolong his life, if not for the purpose of realizing Faust's utopian dream of immortality, at least to ensure that man's twilight may be as bright as possible, delaying the final fall of the mist-shrouded night. And third, to know the biochemical processes underlying the etiology and pathogenesis of the three great groups of diseases: neurosis, biosis, and sclerosis, that is, functional, organic (including the infectious), and degenerative diseases, for this is the only way we can get to know them, prevent them, and cure them.

To solve these problems, contemporary medicine has already started on seven different tracks:

It is using biochemical, physiotherapeutic, and dietetic resources to strengthen normal human physiology and organic defenses as the best means of converting the body into a fortress, walled against the invasion of disease and capable of resisting

all sieges or of expelling any invader that may succeed in penetrating into the human citadel.

It is applying ecology in providing man with the most suitable environment to safeguard and improve his health, his well-being, and his culture. Pursuing this end, "portable" environments with adequate food and artificial climate have already been created to enable man to travel to the polar zones, the tropics, or in cosmic space, carrying with him his most favorable ecologic environment.

It is combating the causes of disease—germs, toxins, traumas, stresses, radiation, noxious foods, noise—by trying both to prevent them and to eradicate them as they spring up. Our aerocosmic age has brought about the vertiginous development of a new discipline, space medicine, whose greatest value lies in its findings not so much about man's life in cosmic space as about man's normal physiology.

It is synthesizing new drugs to treat "new" diseases (alterations of the collagen system, the ABD syndrome, Korean icterohemorrhagic fever, radiation disease, antibiotic-resistant infections) native to our times, which are man's penalty for the progress of civilization.

It is using new drugs to treat "old" diseases, replacing the bow and arrow type of medication with weapons of "atomic" scope, such as antibiotics, ataraxics, new vaccines, and the more recent diuretics and anticholinergics.

It is discovering different techniques to study those processes of the body still unknown, in the hope of forcing the organs to reveal their secrets in that mysterious language of theirs that is translated into data, figures, microstructural images, and laboratory reactions. These new techniques have also revolutionized the teaching of medicine and medical communication by placing at their service a dazzling armory of electronic resources.

To diagnose diseases still unknown, it is seeking and investigating new substances, such as the experimental cancerogenic agents, the new cholesterol solvents, and the hallucinogenic drugs that are now being used in mental diseases and that may disclose some of the secrets of schizophrenia and perhaps even the roots of artistic genius.

THE DANGER OF CREATING A MYTHOLOGY OF SCIENCE

These, then, are the historical challenges that medicine faces today, challenges that must be met not by philosophers, priests, or visionaries as in the past, but by scientific investigators, men who have been driven into *research* by their *search* for truth and for their own personal destiny.

If that brave infantryman of Medicine, the practicing physician, must daily provide prompt answers to the problems of the moment of his patients, since their lives may depend on his decisions, the medical investigator must provide more permanent answers to general medical problems, answers in which he must even anticipate the future. If clinical medicine must answer individual challenges of a temporal nature, medical research must answer universal and eternal challenges.

Never before have investigators had at their disposal so many and such good tools and equipment and so much economic aid with which to face medical problems. Even the popular support that in every period in history has invested great heroes with a golden halo is now accorded to investigators.

But the present formidable advance of science entails the danger of superimposing a mythology of science over contemporaneous scientific thought, a danger that, like a storm cloud, is soaring across the sunlit scientific panorama of our time.

What do I mean by mythology of science?

Well, we know that ancient mythology was characterized by a monstrous overestimation of the hero's powers. The mythological hero was credited with supernatural powers, superhuman energy, unparalleled bravery and extraordinary determination, which enabled him to do anything, to overcome everything, to master all things and beings. More impressive than his exploits was the extraordinary fact that he always faced impossible enterprises with the foreknowledge that he could accomplish them.

The hero's weapons were equally overevaluated and had an importance equal to that of the hero himself. Siegfried's sword, Apollo's arrows, were considered invincible.

Finally, each individual triumph of the hero was so overestimated that his final goal was lost sight of. Each successive victorious step was confused with the accomplishment of the hero's original mission. The labors of Hercules became more important than their original motivations; Ulysses' voyage because of its epic magnitude eventually obscured its original purpose: to return to the loving arms of Penelope.

However, mythology was not made only by the poetic bards in the days of Homer and classical Greece. Mythology has continued to be made all through history, even in medicine, and the result has always been the same: the myths, which are only projections of man's hopes and fears, just as legends are history distorted by the imagination, eventually supplant reality. This is what happened with the myths created around Imhotep and Aesculapius. Imhotep actually existed three thousand years before Christ, but twenty-five centuries later he was a god; and Aesculapius started as a prince of Thessaly and ended centuries later as a god of medicine venerated in hundreds of temples throughout Greece.

Man to this day continues to create mythology, because he simply must have heroes to protect himself against the tedium of life, and because he still needs to project his hopes and fears onto extraordinary beings and objects. Recently, the psychologist C. G. Jung demonstrated in a magnificent study that "flying saucers," whatever their physical origin, are crystallizations of a popular psychic projection of the myth that intelligent forces do exist in cosmic space. In such sense they are a "modern psychological myth."

Likewise, in medicine a dangerous mythology of science is now being created. This mythology has the same characteristics as the mythology previously mentioned, that is to say, overevaluation of the almost supernatural power of the hero—in our case, the scientific investigator; overevaluation of his weapons or tools—in our case, technology; and confusion of his partial triumphs—the conquests of applied science—with his final goal, which is a better knowledge of nature and of man so as to be able to protect his health and prolong his life.

An example of the first characteristic is the unfortunate popular publicity nowadays given to the scientific investigator, which leads to the belief that every investigator who has developed a new substance has already conquered the disease for which it is intended, or that each new drug—ataraxic, steroid, toxoid, antibiotic—

is the definitive therapy for a disease. An example of the second characteristic is the almost religious worship of all technological advances in electronic instrumentation, laboratory techniques, and the rest, while the object for which they are destined often takes second place. An example of the third characteristic is the recent overestimation of certain drugs, for instance, ataraxics, which are really only a key to unlock a few doors in the unexplored castle of the psychoses.

We must be on guard, lest this mythology of science grows roots and takes the place of reality, lest excessive faith in the investigator's invincibility and the power of his technological weapons and excessive glorification of practical scientific victories end in diverting science from its true path. The frequent sight of his countenance mirrored in the pools of technological progress might make the investigator forget to keep his eyes fixed on the broad sunny horizons that are the true objective and ideal of science. Each of the investigator's triumphs is a step forward in the march of progress, but technology, that is to say, applied science, can never replace basic science, wherefrom flows, like water from a spring, the clear philosophical stream that guides man on his way through history.

Above all, it is necessary to banish the growing tendency to experiment for the sake of experimenting, without a definite philosophical purpose. Though Claude Bernard recommended hanging up imagination with one's hat upon entering the laboratory and retrieving them both only upon leaving, he himself never failed to exercise his *scientific* imagination, a luminous and almost poetic vision of what he wished to attain by his experiments, which he wore on his brow like an invisible hat from which he was never separated.

Experimentation is the foundation of science, though it is not its *raison d'être*, as devotees of the mythology of science claim. It must at all events be guided by ideas, by hypotheses, by an intuitive vision of the ideal goal that it wishes to reach. Should it not be so guided, the now developing hypertrophy of "pure" experimentation will ultimately lead to atrophy of the investigator's imagination.

The best remedy against the danger of mythology is for all those dedicated to science continually to remind themselves with humility, as the Carthusian monks continually remind themselves of death, that both the investigator and technology have their limitations. Although the answers we have found to concrete problems up to the present have been dazzling, the greatest problems still remain unsolved after some six thousand years of written history.

The mythology of science, that is, excessive credence in what science can do for humanity, may continue to grow among the general public, but I feel sure that we scientists will try to maintain a human nonmythological attitude, because we know that without it there can be no real progress in the field of science.

I believe this because, quite apart from the dazzling mythological armor in which the public arrays the investigator, the investigator does possess certain virtues, invisible to the public, that give him inner stature and in which reside his true greatness and that of his work.

THE SEVEN VIRTUES OF THE MODERN SCIENTIFIC INVESTIGATOR

Everyone knows the technical resources today available to the investigator in accomplishing the formidable task entrusted to him. I shall not quote statistics, for

though I sometimes use them I dislike them strongly, just as I hate umbrellas, yet use one when it rains. Instead, I shall mention the spiritual resources of the investigator, his seven capital virtues—as I called them in a recent study on Carlos Finlay, “The Pasteur of the Americas,” discoverer of the mosquito’s role in yellow fever—virtues which hold the key to his greatness and the justification for looking forward with optimism to medicine’s response to the great challenges of our time.

History, including that of medicine, is made by men, and on the number of spiritual carats such men possess depends the possibility and magnitude of their triumphs.* But even in the clean, fresh climate of science, one needs a purpose, a method, a spirit. I have already spoken of the purpose and the method of contemporary medical science. Its spirit is that of the men who are today making that science. Benjamin Franklin said, “All mankind is divided into three classes: those that are immovable, those that are movable, and those that move.” Scientific investigators are those who *move*, who do things, who express their greatness in actions of vast consequence to humanity. On their virtues depends the progress of science. The best scientific machine or instrument, the most perfect laboratory, the most advanced statistical methodology, the latest electronic advance, all these would be worth nothing without the man who, in the laboratory, clinic, office, or library, contributes his life so that the stately chariot of medicine may move forward. Without these men no history of medicine could be made, just as no good building could be erected without pillars to support its foundations.

Foremost among the investigator’s virtues I place *goodness*. A castle is as strong as its foundations. Every great man, every great investigator, begins by being a good man. The humaneness of daily life must overflow into the humanism of the investigative genius. In no profession as in medicine is it so important to be a good man. “Science without conscience is nothing but baseness of the soul,” said that lapidary of eternal truths, Montaigne. The true investigator is an indefatigable galley slave of medicine, who, renouncing the glitter of easy success and tempting profit, chooses the troubled seas of investigation instead of the calm harbors. Modesty is usually the inseparable companion of such goodness.

The second quality is *greatness*. Greatness is simplicity. This has been so from Hippocrates to Fleming. A greatness that is an aristocracy of the spirit is the only sovereignty people can accept without relinquishing their own sovereignty. Greatness in the true investigator is to go through life doing with a simple spirit things that may benefit his fellow-beings, building a mighty pyramid without losing his childlike innocence of soul. It is to remain indifferent in the face of indifference, to have faith in his convictions, even during the interminable night of failure, certain that the dawn will come. It is to accept life—as did El Greco in Toledo—as splendor and radiance, lighting the shadows of his laboratory with his own inner light.

Sometimes *genius* is added to these qualities. While in art knowledge is spasmodic and non-accumulative, in science it is systematically accumulative and keeps on replacing itself. A Botticelli, a Cervantes, a Beethoven, each in himself is a complete cycle who needs no predecessors and leaves no school. Hence a work of art—symphony, statue, painting—is immortal, while the life of a scientific work—report,

* Science has progressed because it stepped from what was its basis in the past, that is, qualitative impression, to its present basis, which is quantitative measurement. If to make art is to create, to make science is to *measure*.

address, lecture, or research—is ephemeral and always liable to be superseded by a more recent work.

In art, genius is the rebel artist who creates his own universe in its entirety; in science, genius is the rebel investigator who excels his universe by applying the spark of his intuition to already existing knowledge. Genius is intuition, but it is also logic in the service of a fervent vocation, displayed at the right time and in the proper place. Besides quality, genius is also quantity, as borne out by the prolific work of Paracelsus, Harvey, Hunter, Freud, and Fleming. The true genius rarely makes a single discovery. The great investigator, generally, makes many.

Add to that the *spirit of inquiry*, in which experimenting is the indispensable complement to theorizing thought and not a means for exploding sensational intellectual fireworks. Patience in waiting and quickness in thought distinguish the investigator. Often the genius' intuition flashes suddenly, but the true investigator will, if necessary, spend years in verifying his spark of truth, reconciling noble haste—haste, eternal companion of the creator—in his endeavor with patience in its confirmation.

Lucidity is another quality indispensable in the investigator, because the style is the man, or in other words, man in his human quality is his style. In his tribute to Claude Bernard, delivered in Paris on the 3rd of April, 1879, the great historian Ernest Renan said of Bernard's style words that could be applied today to any investigator: "Human intelligence is a whole so well united in all its parts that a great mind is always a good writer. The true methods of investigation, given a firm and healthy judgment, embrace the solid qualities of style. . . . The standard of good scientific style is lucidity, perfect adaptation to the theme, complete forgetfulness of self, absolute abnegation. That is also the standard for writing well, whatever the subject. The best writer is the one who develops a great subject and forgets himself in order to allow his subject to speak for itself. . . . He uses words like a modest man uses his clothing, to cover himself. . . . He thinks, he feels, the words flow. . . ."

The style of the good investigator sparkles with the diamantine brilliance and limpidity typical of the man who knows certain things thoroughly and wishes to explain them so that everyone will understand. Reading great writers, like Erasmus and Juan Luis Vives, one realizes that style is like the acrobat's tights, which cover his figure without concealing it, like a close-fitting glove on the hand of thought. Fleming's reports are like a transparent window through which one can see unmarred by a single mote of dust the wide prairies of his thought. The creed of the true investigator is fidelity to the truth, and that means not only to seek it and serve it, but also to express it in language intelligible to everyone. Finlay, Cajal, Freud, Fleming, Osler—these men allowed no hyperbole or redundancy, no exaggeration of concepts, to creep into their prose and cripple its scientific probity. Lucidity in style presupposes lucidity in thought, and this in turn implies clarity of spirit. A clear style, like a clean windowpane, indicates that the owner does not fear inquisitive eyes. The investigator's prose must be fluid, fresh, and crystalline, like the water from a mountain brook.

The investigator is a true *patriot*, because he is a living part of the historical conscience of his epoch. He can look at the problems of his country and of his time with eyes both critical and loving, for the true lover does not respond blindly to Cupid's darts but loves his beloved though his eyes are wide open to her defects.

The investigator's homeland grows with each of his discoveries, until it becomes a universal homeland not to be found in any geography book. The investigator is the *homo universalis* of our time and helps to promote the health of all people on our planet. He can sometimes, without leaving his home or country, delve so deeply that he reaches universal roots, preferring vertical profundity to horizontal spread, which guarantees his universality in space and his immortality in time.

Finally, the investigator possesses *universality*. In the address already quoted, Renan said, "Glory has something homogenous and identical. Everything that vibrates produces it. There are not various types of fame, any more than there are various kinds of light." All glories derive their rays from the same source. The investigator's glory is universal and clothes with honor all who can admire an ideal and dedicate themselves selflessly to it.

L'ENVOI

In these times of atomic and cosmic terrors, it must be remembered that when all the mechanical marvels of the age have been replaced by others, the example of the selfless investigator will endure unshaken as an inspiration to man and peoples and as a memorial pillar for all time.

On such virtues is founded my optimistic readiness to believe that the mind of the investigator, like a many-colored glass dome shedding its radiance on all fields of human knowledge, guarantees the unceasing progress of human effort.

Other speakers here are better prepared than I to describe and extol the vast intricate technical resources, instruments, equipments, and institutions now at the service of science and of the investigator. I, like a flower-laden botanist emerging from the woods, would only like to pin in your lapel a sprig of seven blossoms: goodness, greatness, genius, spirit of inquiry, lucidity, patriotism, and universality. On those seven ineffably sweet-smelling flowers is founded my belief that the investigator will solve the greatest scientific challenges that humanity has ever faced by writing opposite those dark pages of technology, which record hydrogen bombs, war-provoking missiles, and other infernal machines, those glittering pages that can be written only by the spirit of man.

Introduction of Doctor Austin Smith

FELIX MARTI-IBÁÑEZ

No pageantry ever written on the past history of antibiotics has glittered as much or has had the same impact and drama as the *living* history that is today being made and written about in newspapers and journals.

We have had a glance at the vast panorama of the great challenges in medicine throughout the centuries. Let us now look at the present, and meet the men who are challenging the challenges and answering them with their ideas and their wits, their courage and their dedication. Their words will paint the vast canvas of what society and scientists are doing today to answer the great unsolved riddles of medicine.

The remarkable progress made by medicine in a not too distant past was instrumental in the creation of the pharmaceutical industry and stimulated it to produce in vast amounts the drugs that were being discovered and that were vitally needed the world over. The pharmaceutical industry, in turn, has paid back its debt to medicine by developing many of the new drugs that are now being used in this country. Through the most authoritative voice of the pharmaceutical industry, we will learn about their problems, their answer to the challenge of new drugs to treat old and "new" diseases, and their dreams for the future.

We are most fortunate to have as guest speaker the former editor of *The Journal of the American Medical Association*, now President of the Pharmaceutical Manufacturers Association, Dr. Austin Smith, who is very well known to all of us. To his many endeavors and achievements, Dr. Smith adds such virtues as goodness, kindness, and courage, and to me goodness, kindness, and courage are the three greatest qualities in a man, and especially in a physician.

Dr. Austin Smith will now speak about "The Challenge of New Drugs to the Pharmaceutical Industry."

The Challenge of New Drugs to the Pharmaceutical Industry

AUSTIN SMITH

President, Pharmaceutical Manufacturers Association, Washington, D. C.

If I wanted to be facetious I would say that the challenge of new drugs to the pharmaceutical industry lies in the drugs to be discussed at this annual symposium on antibiotics. With more than 200 scientific papers and almost 400 authors, where else does one need to turn for almost a full-time challenge, especially in view of the fact that many of the terms used are new to a large part of the professional and scientific bodies, which presumably some day will employ the chemicals bearing these names. However, I cannot be facetious, as this symposium will reflect, as it progresses, only one part of the possibilities of modern research—and at the same time part of the hope that lies in store for the sick. Yet, how short is the time period since the word “antibiotic” came into prominence. And how broad is its application as one thinks of diseases ranging from infections to cancer, from bacteria to viruses, from cellular metabolism to enzymology, from disease prevention to food additive. Nevertheless, antibiotics while increasingly important represent only one phase—even though it is an important one—of modern research and therapy.

Perhaps, then, here is the first challenge facing the pharmaceutical industry, namely, how to bring into proper focus the importance of any one phase of research in the industry. Immediately this implied question leads to others, since such an educational process would involve management, production, sales and research in a firm, professional groups, such as pharmacists and physicians, patients, in fact, the community as a whole. While the manufacturer of drugs must set his sights to determine the feasibility of making certain drugs, the health professions must be informed concerning the usefulness of the drugs, the patients must appreciate their responsibilities and the limitations of drug therapy, and the community must prepare to meet the problems created by today’s lifesaving measures. It may seem strange but the drug industry has created problems for itself by helping to save and prolong lives, but so have other members of the health team. Under such circumstances, the most difficult challenges confronting the drug industry are not the unconquered diseases but the problems that are created as diseases are conquered. If the drug industry had not been so successful in its missions, it would not be confronted with today’s many perplexing problems. Which seems to be a strange reward for success.

Until recently, the unexplored areas of scientific attainment raised the most discussed problems for drug manufacturer and physician to ponder. Infections, deaths in early childhood, lingering illnesses, painful deaths were only a few of the challenges for the health forces. One thought primarily of pneumonia, meningitis, cardiovascular diseases, cancer, muscular disorders, and other serious ailments. Then modern chemotherapy became more than a dream, daring surgery became a

reality, good nutrition became common practice, and new concepts of the control of diseases and symptoms became practical. As a result, infant mortality decreased, the life span lengthened, periods of illness became shorter, rehabilitation converted the disabled to the able, and countless lives were saved and countless hours added to the usefulness of men and women. Unquestionably, mankind has benefited from these medical advances and will benefit even more in the future, since the end is not in sight. Unquestionably also, this has meant great economic savings for the ill who returned to work sooner and free of crippling complications. Unfortunately, it also has meant the development of a series of problems, which society apparently is not yet prepared to solve. In other words, scientific progress has outstripped sociological progress and, as a result, it now risks being penalized for its successful realization of scientific goals.

As reports of new miracle drugs—as they are labeled publicly—were revealed to scientifically trained audiences, news of this reached the ears of the public. As time passed, the public demanded more information and a new type of reporter, the medical science writer, came into prominence. Torn between the desire to report news and the desire to make news, some men and women in the field of journalism sometimes were in need of guidance, which at times was not forthcoming. As a result misunderstandings arose and, for that matter, continue to arise. This was a natural result, since much of the subject matter was difficult to understand even by the scientifically trained, let alone by those without scientific backgrounds. Here lay an opportunity—and here still lies opportunity—for leadership and helpfulness on the part of the health forces, particularly those who contribute to health news by drug production, surgical skill or diagnostic acumen. Here, then, is a challenge for the drug industry, a challenge that arose, in part to plague it, because of its success.

As new drugs became available, a need for disseminating helpful information became paramount. As researchers reached farther afield, more new discoveries were announced, and as competitive forces so typical of the United States came into play, more and more drugs appeared. At the same time, deep concern was raised about how to keep physicians informed concerning the availability and usefulness of these substances. So it was to be expected when advertising methods and new educational techniques were employed to capture and hold the attention of the medical practitioner. Much of this was, and still is, unquestionably good. Some may be considered unnecessary but who is to say what is necessary and what is unnecessary if it means the lessening of suffering or the saving of even one life? One thing is certain, though; some people are saying there is too much activity in the promotional field. Sometimes they are confusing, I think, promotional efforts with educational efforts. Be that as it may, this fact remains: The drug industry has created a problem for itself by successfully producing new lifesaving drugs about which there is a real need for quickly disseminated and revealing information. Should the industry not try to describe the availability and usefulness of these drugs because of failure of others properly to appraise the information? I do not think so. Or, should the industry decide not to make available newer and better drugs because not everyone knows all he should know about the existing ones? I do not think so. I know I would not want to be the one to make such a decision if I had at my command a new treatment measure for the sick. Well, it's obvious that

here is another challenge for the pharmaceutical industry, again of its own making because of success in its laboratories.

As new drugs became available and public as well as professional interest in them increased, speculation seemed inevitable about how the drugs should be publicized, utilized, even disseminated. Philanthropic groups, legislative bodies, enforcement agencies, medical and pharmaceutical groups, even civic organizations, began to display an eager, if not always well informed, response to this developing general interest. First one, then another, offered a voice until today there is public clamor for action—just what kind of action is not certain but it seems to be based on a generally unproved concept “I can do it better than the experts.” In this instance the experts, to my way of thinking, are people and a way of life, the people being the practitioners, manufacturers, and medical researchers, the way of life being our system of free, openly competitive, enterprise. Somehow it reminds me of the many armchair coaches who can always play a football, baseball, or hockey game better than the professionals, and the backyard gossip who knows best how to diagnose and treat an illness even though she never had an hour of formal medical training.

Part of this clamor is based on a resentment to being ill, part on a reluctance to pay for something not normally associated with pleasure. Personally, I think it costs much more to remain well than to be treated for an illness. For example, every day we buy food or clothing or pay rent. This is part of keeping well, but how often in a lifetime does any one of us pay for a severe illness? Nevertheless, sickness seems to have more of an emotional appeal than health and we find as a result the would-be-planners of our lives proposing a system of control over remedies for illness, an occasional occurrence, that goes far beyond proposals for health maintenance, an everyday occurrence. However, this is not the time nor the place to discuss the motives of such planners; I only mention them because their activity, and success if any, rests solidly on the success of the drug industry and other members of the health forces. If we had not been successful in combatting disease, there would be no bricks and mortar for the foundation on which our critics rest their arguments for charge. This, too, is a penalty of success and it offers a challenge for the industry to meet and not ignore.

As youngsters we were fascinated, at least I was, by fairy tales, such as “Jack the Giant Killer.” Emotionally we lean toward the small one, the underdog. Sometimes people never emerge from the emotional ties of childhood. An example, I think, is the person who believes something is wrong because it’s big. Is a business to be frowned on because it is big? Or an industry? Or our children because they’re several inches taller and several pounds heavier than preceding generations? Is an apple grown in a 400 acre orchard worse than the one grown in the 10 acre orchard? The drug industry is now “big business” and as such will be subjected to searching scrutiny for years to come. To those who do not understand such things, this will suggest an element of questionable practices. For such people, an educational program is in order. So, another challenge for the drug manufacturer stems from the fact he can successfully find and market drugs that are so useful and so desired that they put him in the “big business” classification as he turns out the amounts necessary to meet the demand.

Similar in principle, perhaps, is another unfounded suspicion that drug manufacturers cannot be depended on to provide correct or sufficient information with-

out government intervention. I can only say, because of shortness of time, that in general I believe those who deal with the human life become increasingly respectful of their responsibilities whether they be drug manufacturers or physicians. Nevertheless, herein lies a problem of growing significance, which offers a troublesome challenge for the drug industry. This challenge will not disappear by turning one's back on it; in fact, if unmet it might increase until it becomes engulfing, not just challenging.

Now, what about the problems associated with lack of physicians, lack of scientific personnel, lack of teachers, in fact, lack of schools? Some people are critical of the drug industry because it offers modern laboratories, modern research tools, and some comforts of life for the researcher! Is this wrong as long as mankind benefits? Should the blame be placed on the manufacturer or on a system that does not provide equal opportunities elsewhere? It seems to me we must look beyond a readily available whipping boy if we are to meet our obligations within the framework of modern society. Criticism is good if it is constructive but if it's only intended to be face-saving or act as a camouflage it serves little useful, perhaps much harmful, purpose. Yet we who are interested in the drug industry cannot turn our backs on this problem just because it is not solely of our own making. This, too, is a challenge for the manufacturer of new drugs. Here is where community leadership will become important and will be helpful.

Well, I could go on listing some of the challenges today facing the pharmaceutical manufacturer in his role as drug manufacturer, as business man, as a special member of the health team, and as a good citizen, but time for me I am sure is about at an end. So, I would like to summarize what I have been discussing by saying that perhaps the drug industry has created more problems than it has solved—not necessarily through any fault of its own. It has added immeasurably to the comforts and longevity of man—but it has helped create a pool of millions of people in this country alone who are too old to work, at least according to the concepts of modern society. It has added countless lives to our population—and at the same time created a need for more doctors and other members of the health forces. It has helped develop a pool of biologically active and able older people, but it has not provided at the same time more money for those on social security. It has reported the availability of curative, almost miraculously so, chemicals—but it has only stirred the public to accept casually the improbable and to demand the impossible. Or, as I have said before: The drug industry has helped advance medical science beyond society's ability to cope with it. It has outrun its pursuers in this respect. In fact, maybe it is too far ahead of itself. However, this is no reason for crying "halt," any more than one would cry halt on modern plane production because runways are inadequate. Build the planes; the runways will follow; and so it is with drugs. Produce better drugs; the other problems will be solved. My only point in this respect is threefold: First, let everyone realize that health is a personal and civic responsibility, not just one industry's, or the government's, or one or two professions; second, because an industry or a profession outstrips others in its efforts is no reason for it to be singled out for punishment; and third, no one industry or profession in the health field can stand alone today, since its efforts exact such a profound effect on others, and in turn are so deeply affected by the actions of others. Here, then, lie some of today's greatest challenges for the makers of new

drugs, which go beyond the search for cures and include the control of diseases plus participation in the solving of the problems that may arise as diseases are eradicated. Here is where science and leadership must become one, and here is where the most effective work of a combined health team can be revealed. Here is where medical care can continue to provide, as it already has done, a greater contribution to mankind than even the industrial revolution of a few decades ago.

Introduction of Doctor Maxwell Finland

FELIX MARTI-IBÁÑEZ

Ever since his graduation from Harvard Medical College 33 years ago, the professional history of Dr. Maxwell Finland has mirrored the history of chemotherapy in the world. His tireless efforts, his selfless devotion to his work, his scientific austerity, integrity, and honesty, the three basic virtues of the true investigator—all these have made Dr. Finland one of the greatest international authorities in the field of clinical chemotherapy.

As you know, under the Food, Drug, and Cosmetics Act of 1938, no new drugs can be made available to physicians in the United States without a thorough clinical and experimental study to demonstrate their safety. This safety of new drugs must first be recognized by investigators of the highest scientific caliber, to whom is entrusted the meticulous clinical investigation of such drugs.

In this great white army of investigators, Dr. Finland is what Sir William Osler would have called a "Captain of Medicine," and it is he, the Associate Professor of Medicine of the Harvard Medical School and Associate Director of the Thorndike Memorial Laboratory, Dr. Maxwell Finland, whom I now have the privilege to introduce. He will speak about "The Challenge of New Drugs to the Clinical Investigator."

The Challenge of New Drugs to the Clinical Investigator

MAXWELL FINLAND

Associate Professor of Medicine, Harvard Medical School; Associate Director, Thorndike Memorial Laboratory; and Physician-in-Chief, Fourth Medical Service, Boston City Hospital, Boston, Mass.

When I was first approached with the request to undertake the task now before me, my first reaction was clearly and, I thought, unalterably in the negative, as I was far from convinced that I was the person to do this, nor did I consider that this would be the proper time and place to air my views on this subject. Indeed, I suggested several outstanding persons who by both word and deed had already demonstrated their distinction in the field of clinical evaluation of drugs, who were much more suited to the task and could do it more effectively and authoritatively. However, the chairman of the program committee, not convinced by my reply, and in the manner nowadays customary among those in the unenviable position of having to get together some people to contribute within certain preconceived and prescribed areas that fit into their particular program for a meeting already scheduled, called me long distance and reached me at a time when I was preoccupied with other matters. At the moment it seemed easier to say "yes" and get on with what I was doing than to spend the time arguing—so I accepted, which, of course, is what the program chairman always hopes will be the case.

As the time of this meeting approached, it was difficult for me to formulate just what to say and how to say it. It seemed obviously appropriate to review the methods of setting up a therapeutic trial, how to choose the proper personnel, how to obtain, formulate, and evaluate the background data, pick the proper places and types of clinical material, how to record the observations, how to know when there are enough observations to provide a definite answer, and then how to analyze the observations so that they provide meaningful answers. However, this, as I have already intimated, has already been done by a number of well-qualified investigators both in this country and in Great Britain, and it would be presumptuous on my part to tackle this type of presentation. Moreover, I had to assume from the fact that the idea of asking some of those workers to accept this assignment was rejected that this was not what the program committee had in mind.

Fortunately, I was assigned no special area that I was expected to cover other than the broad one of clinical investigation. My own experience in this field has extended over the past 30 years; it was concerned, at first, primarily with the specific therapy of pneumonia and then with the general area of therapy of acute infectious diseases. This has inevitably brought me face to face with the difficulties arising out of the availability of rapidly increasing numbers of new drugs, the products of the logarithmic growth phase of a highly prosperous industry that has succeeded in recruiting some of the country's best biological scientists and chemists who have devoted most of their efforts to this task.

The availability of clinical facilities and of persons trained in the clinical evaluation of these new agents has lagged so far behind that it has been possible to subject

only a very small proportion of the total new drug output to any but the most perfunctory type of clinical trial. Moreover, the highly competitive nature of modern industry in this country—aided and abetted in no small measure by the fast-working and aggressive members of their sales forces, advertising industry, and the financial wizards behind them—has made it most difficult for the few clinical investigators who do expend their efforts in this field to be heard above the din and clatter that is so frequently raised even before they have an opportunity to get together the data on which to base an opinion and before they have adequate time to analyze, check, and evaluate these data and present them for critical appraisal among their peers through publication in reputable clinical and scientific journals.

ROLE OF CLINICAL INVESTIGATOR

It may be putting the cart before the horse to talk about the role of the clinical investigator in the discovery and development of new drugs without attempting to define what is meant by a clinical investigator. However, this, as we shall see, can best be done by defining his function.

Perhaps the most important and the most intellectually satisfying function of the clinical investigator is to initiate ideas that lead to the possible discovery of new agents or to the application or adaptation of known or readily available agents to previously unsuspected uses. This can come only from careful clinical observations with meticulous attention to detail, and requires a keen and patient observer, or it may come from chance laboratory observations in the course of functional studies carried out during an evaluation of the diagnosis, functional status, or progress of the patient.

An outstanding example is the development of modern treatment of pernicious anemia. This was based in the first instance on careful documentation of the dietary histories of patients and the recognition of the possible conditions in the patient under which remissions occurred and then putting them to the test by applying them to patients in relapse. Certain other important factors were necessary to bring out this discovery and to hasten its more definitive application. One was the recognition of the role of the reticulocyte as a measure of new red blood cell formation and maturation. Although it may have been possible to bring about improvement in patients without this test, the full development of the potentials of this form of treatment and its application and extension to other types of anemia were certainly hastened by the use of this simple laboratory test.

A second role of the clinical investigator is to aid the laboratory scientists in initiating new agents in needed areas or in developing new drugs along lines of research that may already be developed but which the keen and knowledgeable clinical investigator recognizes as a possible answer to questions raised in the management of his patients. The development of the sulfonamide drugs, their application to the treatment of human infection, and indeed the whole course of modern chemotherapy is due in large measure to the thorough understanding of the nature and course of streptococcal infections and to the recognition of the significance of changes wrought in the first few patients with such infections to whom the first active members of this series were administered. The exploration of these drugs and the developments and improvements of this group of agents could not have

been made and been advanced without careful clinical observations at each step, which sifted out the more active and less toxic agents. These advances, too, were accelerated by the development of simple methods of quantitating the drugs in body fluids and tissues.

Although the chemists and pharmacologists working in the laboratories have made great progress toward the correlation of chemical structure and function, it still remains a fact that the major discoveries, at least in the field of infectious diseases, and particularly among the antibiotics, have been made empirically and without any reference to the chemical structure of the final product; this only followed, and sometimes much more slowly, after much more tedious efforts than were required to obtain the active product and determine its full value. Thus, millions of patients were cured and thousands of lives saved by the major antibiotics now available before their chemical nature was known, or even suspected, since many of them revealed new structures not previously known or recognized.

Even in the development of sulfanilamide and its derivatives, it could hardly have been predicted that sulfanilamide was the active principal of Prontosil and that sulfanilamide would produce severe hemolytic anemia very frequently, but that the addition of certain moieties would result in reducing this toxic effect but bring out others, such as the severe nausea and vomiting from the pyridine derivative and the marked sensitizing effect of the thiazole derivative. None of these factors were predictable and they could be recognized and evaluated only by the clinician.

A third and important function of the clinical investigator is to explore the possibilities of entirely new agents and principles developed by the laboratory scientist, for, no matter how profound and logical the thinking that goes into a new principle or a new product, or how brilliantly successful it may appear to be in the test tube or even in the mouse, the guinea pig, the rabbit, or even the dog, it ultimately has to be administered to patients with illness, and it is on the effects on the patients and on their illness that the success or failure of a new drug depends. Moreover, there are diseases peculiar to man in which all of the aspects of activity can be tested only in the patients, since there are no counterparts or models *in vitro* or in animals.

Here the investigator must have a deep appreciation of the background of the discovery, an understanding of what the scientist has done, what he must require in the way of preliminary information obtainable in the laboratory that might be helpful in anticipating what may happen in the patient. This must be coupled with a thorough understanding of the disease and possible reactions of patients he is to choose as the proper subjects for the initial trial, the proper observations to make in the initial trials, and the proper preparations to make in the event of emergencies that might arise and that he must, if possible, anticipate.

There is no place in such a scheme of things for the brash and the ruthless or for unbridled trial and error, for one must always think first and foremost of the patient who is the subject of the clinical trial, to avoid, as far as possible, doing any harm. This cannot always be done with certainty, so that it becomes necessary to weigh possible harm in the balance as against the possible good that may result to the subject and to others who may benefit from the new agent. This means that one takes risks only when they appear to be justified by the greater possibility of potential benefits.

A fourth role of the clinical investigator is to help in the improvement of drugs already demonstrated to be effective. This may appear to be a simple task, but one in which it may be no less difficult to arrive at truthful and meaningful evaluations, and may also be equally taxing, as in the development of useful and effective parenteral preparations of drugs that are effective orally or in the isolation of the active principles of useful but complex substances.

Finally, the clinical investigator has an important duty in eliminating the useless and ineffective drugs, in exposing unwarranted claims, in accelerating the obsolescence of the useless or the less active and more toxic agents when better ones become available. Here is perhaps the clinical investigator's most thankless and most difficult task, for his is merely a moral duty to his patients, to his colleagues, and to their patients, and this may clash with the more personal vested interests of manufacturers and their associates. Moreover, the clinical investigator must divorce himself of all bias in arriving at his evaluation, for he too may be subject to the same criticism that he may have to offer of the manufacturer, and he should not be unjust to him and to his products. Few are the clinicians and investigators with courage and willingness to undertake this task, particularly those best equipped to perform it, for they may rightly feel that they can more profitably spend their time and efforts in more intellectually rewarding pursuits. Yet this is a clear duty of the clinical investigator, as it is of the manufacturer to accept his findings, if they are properly documented. In the long run, the manufacturer who follows the recommendations of the careful and circumspect clinical investigator and discards inferior products or refuses to exploit them in the first place will ultimately gain the greater respect and confidence of thoughtful physicians and should eventually gain an advantage over his competitors who continue to maintain sales of inferior products at high levels only by virtue of the well-known modern high pressure techniques of "saturation" and brain washing as practiced by their advertising agencies and sales departments.

WHO SHOULD ENGAGE IN CLINICAL INVESTIGATION?

The answer to this question would seem to be self-evident and would seem to follow from what has already been said. However, it is not that simple, for it requires more than just a keen observer who recognizes changes and can record them. The clinical investigator is of course a physician and, as such, his duty is first and foremost to his patient. Like any human being, a physician may develop convictions in the course of his own practical experience or from his interpretation of his own laboratory observations or those made by others in whom he may place great trust. Such convictions may be incompatible with sound clinical evaluations of new principles or new drugs, for the investigator must first and foremost recognize that there is a question to be answered and he must be prepared to evaluate his observations objectively. Even an excellent scientist at the laboratory bench may prove to be hopeless and useless as a clinical investigator in an area in which he has firm convictions and may, as a result, even retard progress through his passionate acceptance and defense of agents he considers useful for patients, but which cannot stand up to the same critical appraisal that this scientist would apply to results he obtains in the laboratory. Such a person, if he is self-critical and will acknowledge

his bias or his unwillingness to make proper and critical comparisons, can nevertheless contribute to our understanding of the drug by making careful observations of agents he considers useful, but should not offer comparative evaluations if he will not make such comparisons objectively.

Some years ago I reviewed the problem of the evaluation of specific antisera in the treatment of various infections and some of the "controlled" therapeutic experiments on which their acceptance was based. Few if any of the conclusions derived from those experiments would now be considered acceptable on the basis of the actual observations reported, logical as the premises seemed to be on which the trials were based. In the course of this review I recalled one of my early exposures to the fallibility of "authority," even when highly placed. At one of the first meetings of the venerable Association of American Physicians that I had an opportunity to attend, Dr. William H. Park presented the results of a well-controlled study of convalescent poliomyelitis antiserum that was carried out in Brooklyn and that clearly showed that the antiserum had no beneficial effect whatever on the course of patients with acute poliomyelitis nor on the amount or severity of the paralysis that resulted. I was then impressed by the mixed motives that sometimes arise in the laboratory scientist when confronted with the adverse results of clinical investigation. It was none other than the great Dr. Simon Flexner, then head of the Rockefeller Institute, who made the following statement in discussing Dr. Park's paper: "There is a consensus . . . that the use of convalescent serum does no harm. Since it cannot be affirmed that, in the individual case it does no good, should its use be withheld? This is a question to be answered not by a pathologist but by a practitioner." Many a budding clinician, like myself, who was hoping to bring a reasonably strict "scientific attitude" to bear on some of his clinical problems felt quite disarmed by these remarks. In this instance, there was no financial gain involved, such as might influence a decision or opinion of a manufacturer, but one could not help but get the impression that there was a personal vested interest of the scientist in a discovery with which he identified himself. It is most unfortunate if the scientist or the clinical investigator is put in a position of having to defend a thesis rather than interpret facts as they are.

A different situation currently exists in relation to the use of adrenocortical steroids in the treatment of infections. A perusal of the literature on this subject shows that it is replete with reports of uncontrolled observations, most of the favorable ones being primarily "testimonials" of allegedly brilliant successes attributed to these agents in what the reporters considered to be hopeless cases, but without reference to reports of similar successes in comparable cases in which the same treatment had been given without result and particularly without reference to the more numerous cases of the same or even less serious ones in which these agents had been used and in which the results were unfavorable. As a result it has become extremely difficult to obtain an objective evaluation of the exact role and usefulness of these agents in serious infections. When that was recently attempted in a double-blind, controlled study, the results were at best equivocal.

The use of adrenocortical hormones in the treatment of severe meningococcal infections, particularly in Waterhouse-Friderichsen syndrome, is now accepted as an essential part of the treatment and is supposed to be the clearest example of a logical and proved application of a specific therapeutic agent. Proof of its value is

offered in the reports of patients who were given these hormones and recovered, but little attention is paid to the fact that definitive treatment of the infection was undertaken concomitantly and that other patients have recovered after such treatment alone without resort to steroids. Few if any writers on this subject fail to recommend them, nor do any teachers question their value. Yet the evidence of their usefulness is very flimsy indeed and the fundamental basis for considering them to be logical has long since been disproved. In the first place, hemorrhage into the adrenals with complete disruption of function is infrequent and not an essential feature of the disease and indeed such hemorrhages are most readily produced experimentally by excessive dosages of ACTH. All present evidence points to continued hyperfunction of the adrenals and production of large amounts of cortisol with high levels present in the blood in such patients, even shortly before death. Moreover, patients who recover have a return to essentially normal adrenal function in convalescence from this disease as from other severe infections. Finally, in successive series of cases treated properly with modern chemotherapy alone, the mortality has been shown to be at least as low as in similar cases in which adrenal steroids have been given in addition.

It is not meant to imply by these remarks that there is no place for the use of cortisone-like agents in the treatment of infections. It is hoped only to convey the conviction that the usefulness of these agents in such infections has not been clearly delineated and that only clinical investigators who have similar convictions could possibly be in a moral or psychological frame of mind to be in a position to undertake any controlled evaluation in this field.

It goes without saying that the clinical investigator must also have a thorough acquaintance with the diseases with which he is dealing and a thorough understanding of the background of the agents he is evaluating and their relation to others that have been used for treatment of the same conditions. If he does not have this background, he may serve only as a tool of the drug house and his evaluation will readily reflect this. His can only be a "test by testimonial" and yield a type of clinical report that some less critical manufacturers are eager to exploit but that the more reputable ones are not too enthusiastic about, for such evaluations do not provide the type of background that is really helpful to them in determining the true value of the agents that they develop after great effort and expense, and how they really compare with others that have similar properties.

In short, the clinical investigator must have a broad background and understanding of disease, be a superior physician, have the proper understanding of and consideration for the patients in whom he makes his studies. He must also be sufficiently well grounded in the basic sciences to be able to understand the nature of the drugs he is testing and the methods and tests used in their evaluation. He must be prepared to take the time and trouble to look after all details meticulously and must be interested in them. He must know what he is looking for and be prepared to recognize and capitalize on the unexpected, should it arise. Finally, he must have curiosity and be unbiased, detached, and critical in the evaluation of his observations. In fact, he must have all the attributes of both the best scientist and the best physician. It is indeed much to ask, but can we afford to entrust the evaluation of new drugs with their great potential for good but also for harm to any other type of person?

It should be obvious that in comparison with the tremendous numbers and varieties of new and improved drugs available for clinical trials, the number of truly qualified and interested clinical investigators is very small indeed. Moreover, the number of places providing adequate facilities for such trained investigators is still grossly inadequate. This is perhaps one of the main reasons why we have so many "tests by testimonials" in contrast to the relatively small number of really useful and critical evaluations of new therapeutic agents. Moreover, among those who are qualified to undertake such studies, the majority are reluctant to become embroiled in possible controversies or to put themselves in the position of undertaking obviously useless projects and expending their limited efforts and facilities on seemingly unimportant tasks merely to disprove obviously unfounded claims in order to provide sound evidence to replace the flimsy and uncritical reports of others.

Under these circumstances, what can be done to make possible the rapid and proper evaluation of new drugs without jeopardizing or slowing down the progress of those who are working to create them? It has been my own conviction, as a physician, that those persons having adequate background and facilities to make proper and meaningful evaluations have a duty as physicians and educators to carry out such studies as part of their clinical responsibilities and within the areas of their competence. Support for the additional personnel and facilities for such endeavors must be provided for these investigators on a continuing basis and without reference to individual drugs, projects, or manufacturers. This, it seems to me, is a proper obligation of the pharmaceutical industry as a whole, which all of its members should support—and generously. It should also be a proper domain for governmental support, since the governmental regulating agencies are interested in reliable information and it would appear more proper for such agencies to support investigators outside of their agencies rather than carry out the actual observations that form the basis for opinions that will, in turn, form the basis for their decisions.

This could be accomplished by establishing and adequately supporting divisions of clinical pharmacology within the various clinical departments of medical schools or in large "teaching hospitals," even those unaffiliated with medical schools but that provide postdoctoral training. The intense competition among such hospitals for interns and residents has resulted in the setting up of programs of education to provide the proper clinical training for our medical school graduates. This has required increases and improvements in laboratory and clinical facilities and the recruiting of better qualified members of the hospital staffs in order for these hospitals to be able to meet the necessary qualifications and withstand the competition. This will inevitably lead to better and more thorough study of patients and the keeping of better records, and should provide the primary prerequisite for proper clinical investigation of new therapeutic agents. If undergraduate students, teachers, and hospital staffs could also be trained in the evaluation of those agents that they use in their patients with the modern scientific methods of conducting controlled studies, we might well be in a position to develop the ideal set-up for proper evaluation of large numbers of new drugs and put them on a sound basis.

Can this be done under such circumstances? My own answer is "yes," for, in

essence, every physician is conducting a clinical trial each time he administers a drug or performs a therapeutic procedure. If this is done with adequate provisions for obtaining background data and for making the observations necessary to determine the effects that this therapy produces and if the results are properly recorded, all of the essentials for proper clinical evaluation are already provided. It is only a short step from this set-up to one that can provide all the information needed. Groups of physicians gathered together as teams of investigators could set up programs for evaluation of agents in certain diseases or of specific groups of drugs requiring specialized laboratory techniques for evaluation. This should provide a very stimulating atmosphere for the conduct of postdoctoral education that would really prepare our medical school graduates for a better understanding and appreciation of the value of the remedies they use.

Unfortunately, at the present time, medical education seems to end largely with the awarding of the degree. After that the clinical training is acquired from preceptors, but throughout most of the physician's career, his education about new drugs comes from the literature provided by manufacturers or, perhaps even more so, directly from their representatives by word of mouth. Experience and training such as is visualized for the departments of clinical pharmacology and for the teaching and training hospitals, through investigators who are properly prepared, will do much to return postgraduate education to the medical school and to the qualified members of the medical profession, where it belongs, and remove it from the necessarily biased auspices of the interested manufacturers. However, it is my conviction that all will benefit by such a change in our attitudes toward the clinical investigation of new drugs—the physician, manufacturers themselves in the long run, but most of all the patients, who are our ultimate concern.

Introduction of Doctor William H. Kessenich

FELIX MARTI-IBÁÑEZ

For many centuries the clinical use of drugs for therapeutic purposes had no other control than that of the physician's professional code of ethics. In the middle ages, clinical medicine was more or less regulated by the physician-priest; from the sixteenth century on, it became regulated by a new political concept—the State. In our times, regulatory government agencies have developed impressive techniques to determine the safety of new drugs and to guide in their clinical application, much to the benefit of the physician and the patient.

There is no one more qualified to tell us the fascinating story of how hundreds of new drugs are reviewed by the Bureau of Medicine of the Food and Drug Administration than its own medical director, a distinguished medical investigator and most gifted organizer, Dr. William H. Kessenich, whose words will symbolize the integration of the efforts of the government, the pharmaceutical industry, and the clinical investigator.

It is with great pleasure that I now introduce Dr. Kessenich, who will speak about "The Challenge of New Drugs to the Food and Drug Administration."

The Challenge of New Drugs to the Food and Drug Administration

WILLIAM H. KESSENICH

*Medical Director, Bureau of Medicine, Food and Drug Administration,
U. S. Department of Health, Education, and Welfare, Washington, D. C.*

The discussion of the morning has been focused on the challenge that confronts our society caused by the growing field of new drugs. It is now the turn of the Food and Drug Administration to present its perspective of this problem. Challenge, by definition, is a dare, or an invitation to a test or trial or contest, and it usually carries the connotation of a test requiring a superior or courageous effort on the part of the contestant if he is to prevail. I believe that we in the Food and Drug Administration recognize that our responsibility in the field of new drugs is indeed such a challenge.

The new drugs presenting us with this challenge are defined in the federal Food, Drug, and Cosmetic Act as drugs that are not generally recognized as safe by experts qualified by experience and training to evaluate their safety. The law further provides that such new drugs may not be marketed until there is an effective new drug application showing full and adequate investigations to demonstrate whether the drugs are safe for use.

It is now 21 years since the passage of the federal legislation that contains this definition as well as the section of the law that controls the marketing of new drugs. It was the passage in 1938 of the revised Food, Drug, and Cosmetic Act that first provided for pretesting the safety of new drugs prior to their introduction into interstate commerce. The original law of 1906 had no such control. It is an oft-told, but still dramatic, story of how the tragic death in 1937 of more than a hundred persons from an untested drug solvent resulted in the addition of the new drug section to the proposed revision of the law. With this stimulus, the revised version, which had been delayed by discussion for several Congressional sessions, was soon passed and it is the statute under which we still function.

From the viewpoint of the Food and Drug Administration, this responsibility of regulating new drugs is now 21 years and more than 12,000 new drug applications old. Although at 21 we might consider that the age of maturity has been achieved, we can see only a new surge of growth in the development of our challenging new drug charge.

The philosophical approach to the control of new drugs by the Food and Drug Administration can perhaps best be related to the intent of the law as it was passed. Basically, it is to ensure that adequate safety testing of new drugs has been accomplished before marketing. This is achieved by prohibiting interstate commerce of new drugs until full information has been submitted showing that the drug is safe under the conditions prescribed in the labeling. This is not considered a licensing provision, but is intended merely to prevent premature sale of new drugs that have not been properly tested. The new drug provision does not put the federal government in the business of developing new drugs nor does it require the government to

duplicate laboratory and clinical tests made by responsible manufacturers. It is simply a method for an authoritative review of the tests that have been carried out on a new medicinal agent before it may be marketed.

Most of the pharmaceutical industry is comprised of responsible persons who are guided by sound scientific principles. Even beyond the high motivations and public regard that cause these men to assure themselves that they do not market a harmful drug, responsible manufacturers know well that for purely business reasons there must be sufficient study of their new products to determine that they are both safe and effective. The pharmaceutical manufacturers over the years have developed in the medical profession and in the consumer a feeling of confidence and reliability that they cannot afford to jeopardize by the release of some dangerously and unnecessarily harmful drug, or a drug that is not effective.

The new drug review procedure provides another check that adds to the assurance that these tests are complete and adequate. It further provides an absolute block to those members of the industry who would not maintain the needed degree of integrity.

At the outset, some persons within the pharmaceutical industry felt that the 1938 law providing new drug control would stifle progress, since it was well recognized that practically every potent drug had possible serious side effects. However, the Food and Drug Administration has always weighed the therapeutic value of a drug against its toxicity and has given particular attention to informative labeling to warn physicians against potential hazards. The fact that these fears were unfounded may be reflected in the fact that the period of the past 20 years has seen the greatest growth and development of the drug industry in history—a period that parallels the years that the act has been in force.

Through these years of new drug review, experience has established some guides and principles to be followed in determining the safety of a new drug. Among other things, a new drug application must contain full reports of all investigations that have been made to show whether the drug is safe for use; a full list of the articles used as components of the drug; a full statement of the composition of the drug; a full description of the method used in and the facilities and controls used for the manufacture, processing, and packing the drug; samples of the finished product; and copies of all labeling to be used for the drug.

The investigations made are ordinarily expected to include full reports of adequate tests by all methods reasonably applicable. They should contain detailed data derived from appropriate animal or other biological experiments in which the methods used and the results obtained are clearly set forth. This usually means pharmacological studies in animals and subsequent clinical investigations. The kind and the amount of information required will depend on several factors, such as the nature of the drug and its indication, and must be determined individually for each new drug.

In general, we expect the animal studies to include an acute toxicity experiment, usually an LD₅₀ study, in two or three species by oral and intravenous administration. Depending on the clinical uses for which the drug will be recommended, longer term animal studies may be necessary. If a drug is to be used only once or for only a day or two, then a two to three week subacute study should usually suffice.

On the other hand, if the intended use will usually require prolonged or con-

tinuous administration, then chronic experiments of from six months to a year are probably indicated. The drug is usually given orally to a rodent and a nonrodent species—customarily rats and dogs. It is fed to one group at a high dosage level to produce toxic symptoms, to another group at a lower but therapeutic level; a third group is maintained for control.

Appropriate laboratory studies should be carried out on these animals during the period of feeding the drug, and at the termination of the study the animals should be sacrificed and the organs studied for gross and microscopic pathology. Special additional studies are required if the new agent is an injectable or is intended for topical application.

With the knowledge derived from the animal studies, clinical trials will next begin. Information relative to the site of activity, the type of physiological response, the range of dosage, and the possible side effects will have been provided by the animal experiments. It is even more difficult to predict how much clinical investigation will be needed to ascertain the safety of a new drug. It is impossible to state simply how many patients must be studied. To a large extent the quality of the study means more than purely volume. The most meaningful information is derived from a well-performed clinical evaluation reporting detailed information for each individual case, such as age, sex, conditions treated, dosage, frequency, and duration of administration of the drug; results of clinical and laboratory examinations must be recorded and a full statement of any adverse effects and therapeutic results observed. Since investigators, clinical facilities, and patient material will vary, one can hardly expect to derive all of the needed information on a new drug from one or even two clinical studies. It is difficult at best to get an accurate picture of the clinical behavior of a drug, and recognizing that some investigators are enthusiastic about any new drug while others are hypercritical, it is obvious that a variety of studies from different clinical centers are needed for a fair evaluation. While some clinical investigations should be this detailed, complete with laboratory study, this does not mean that in addition helpful information may not be derived from broader usage, and in fact, this may help approximate the use of a drug as it will occur once it is marketed.

The most frequently encountered deficiency is in reports that are sketchy and incomplete. Reports of investigators that are in the form of testimonials are worthless. The statement, for example, "I used your drug on several patients. It was successful in the majority and there were no significant side effects," is of no value to the pharmaceutical firm in devising adequate labeling for their drug and it is equally worthless to the physician at the Food and Drug Administration who must review the report. For these reasons, some of the most difficult decisions facing the Food and Drug Administration evaluator arise in the consideration of such clinical reports.

Having considered all of the animal and clinical studies, labeling is the next step in the new drug evaluation. By labeling we mean not only the bottle label, but also the literature accompanying the article. A drug must be safe for use as labeled, and thus evaluation of labeling is another vital step in the consideration of the safety of a new drug. The information derived from the animal and clinical studies provides knowledge regarding the indications, dosage, contraindications, precautions, and side effects of a new drug. We recognize that safety as used here is a relative

term since no drug is entirely safe. We must balance the potential benefits against the possible hazards in reaching a conclusion, and the scale on which this is weighed is frequently the sound clinical judgment of the clinician. And here again, in the case of new drugs, the clinician can frequently reach such a judgment only by an appraisal of all of the facts as presented in the labeling brochures.

The processing of a new drug application provides a review of the data pertaining to the safety of an agent. We can control claims for the efficacy of the drug only inasmuch as they directly involve safety. If, for example, a completely innocuous drug were to be offered for the treatment of a potentially fatal disease and the drug were shown to be worthless, we would have to express the opinion that to rely on such a drug would indeed be hazardous for the patient.

While we cannot prevent a new drug application from becoming effective on the basis of unsubstantiated claims unless such can be directly related to safety, the drugs when marketed are subject to the other provisions of the federal Food, Drug, and Cosmetic Act. This includes the section that deems a drug misbranded if its labeling claims are false or misleading in any particular. In regulating drugs under this section, however, unlike the new drug section, the burden of proof in a federal court is on the government.

There are other parts of the review of a new drug application that, though perhaps not recognized by the clinician, are of extreme importance in assuring the safety of the drugs he uses. The manufacturer is required to submit detailed information on the components, the composition, the facilities, and the controls that are used in the production of the drug. It is on the basis of this manufacturing and analytical control that the physician is assured of a medication that is uniform in identity, quality, strength, and purity.

The statute authorizing this review of new drugs has unquestionably given impetus to the growth and development of the pharmaceutical industry and, in turn, to the tremendous advance in chemotherapy. The provisions of the federal Food, Drug, and Cosmetic Act have required the collection of more and more information about drugs and have spurred efforts to improve the pharmacological and clinical techniques for obtaining data. The increased amount of information required has caused more research to be done on drug products and has resulted in the development of new and often improved compounds. In general, the research required to satisfy the demands for safety has led the pharmaceutical industry to the greatest developmental period in its history, which in turn has benefited the whole of medicine.

While we may conjecture that even lacking the control of the federal Food, Drug, and Cosmetic Act, many of the reputable pharmaceutical firms would have performed adequate study to assure the efficacy, safety, and integrity of their products, there can be no doubt that many others would not have merited such a reputation. The requirements of the law serve to assure that there is a basic standard by which all of the industry is controlled. These same provisions serve also to assure the consumer—in this case the physician and his patient—of a reliable, pure, safe, and effective drug supply. It means, too, that physicians do not have to assume the risk of experimentation, but can use new drugs with the confidence and assurance that studies have already been done that have verified their safety.

It has been recognized over and over again that the New Drug Branch of the

Food and Drug Administration has, over these past 21 years, performed an outstanding service and has made a notable contribution to the cause of the nation's public health. The lessons learned in the past point very clearly to the fact that this era of advancing chemotherapeutics will present new and demanding problems that will have to be answered.

The tremendous strides of the pharmaceutical industry, with increasingly larger sums being expended in the field of research and development, coupled with the multimillion dollar research programs sponsored by government funds, can only result in even more numerous and more potent drugs being developed and made available for use. In the face of this picture of progress—of new drugs, capable of tremendous good, but likewise possessing the potential for serious side effects—there is indeed a challenge facing the Food and Drug Administration. It is a challenge requiring a superior effort on the part of this agency to continue to provide the caliber of review of these new drugs that has been achieved in the past. In view of the increasing complexity of the agents, more potent for benefit and more fraught with hazard, the job of maintaining a proper balance becomes a more delicate task.

To accomplish this task there is also the challenge to the Food and Drug Administration to continue to maintain its scientific competence. It will take our best efforts to keep abreast of the tremendous progress of the chemists who develop the drugs and of the clinicians who likewise are developing new and improved techniques for evaluating the drugs in their patients.

There is also a challenge to our administrative abilities to maintain within the Food and Drug Administration and to foster in the industry and among the medical profession a proper perspective in the promotion and marketing of these newly developed products. In the case of drugs, as in no other commodity, the motivating considerations must be the welfare and benefit of society, and not simply the common lure of the market place—financial gain.

To meet these challenges to our society presented by developments in the field of new drugs, and, having met them, to prevail, requires the highest degree of individual as well as collective effort, plus the cooperation and free communication on the part of all concerned with these problems: research worker, manufacturer, clinician, and government. This effort is demanded if we are to meet and conquer the new drug problems of today so that we can look forward to the even greater challenge of tomorrow, that of providing still other new drugs in the conquest of more and more of the serious diseases with which mankind is afflicted.

Introduction of Doctor John J. Curry

FELIX MARTI-IBAÑEZ

We have heard the voices representing different but overlapping worlds of science; the world of the pharmaceutical industry, the world of the clinical investigator, and the world of the government agencies. We shall now close this session by having a glimpse at the world of the practicing physician.

When the last word is said, the practicing physician is the focus of all the combined efforts of the other three worlds we have already listened to. He, that glorious infantryman of medicine, was, is, and will be the keystone of the castle of medicine. The practicing physician must answer, all alone with his patient in every moment of his work, the tremendous challenge of making the proper diagnosis and prescribing the most efficient therapy. On the failure or success of the practicing physician depends the health of a nation. All the efforts to supply the physician with new drugs would be useless if he did not know how best to use them and how to choose, from the vast array of possibilities offered him, the one that will save the life of his patient.

To explain how the practicing physician meets this challenge, we are privileged to have Dr. John J. Curry, practicing physician, specializing in cardiology, clinical investigator, and Associate Clinical Professor of Medicine at Georgetown University School of Medicine. He will tell us what the physician does in the endless search for practical knowledge about drugs with which to treat better his patients.

I have the pleasure of introducing Dr. Curry, who will speak about "The Challenge of New Drugs to the Practicing Physician."

The Challenge of New Drugs to the Practicing Physician

JOHN J. CURRY

*Associate Clinical Professor of Medicine, Georgetown University School of Medicine,
Washington, D. C.*

The remarkable improvement in our nation's health in the past two decades is apparent to even the most casual observer. There has been a sharp decrease in disability and suffering, our life span has been increased several years as the death rate from infectious diseases has plummeted, the need for hospitalization for acute infections has been notably lessened, and the average length of hospital stay has been reduced to about eight days. The introduction of new and improved drugs is responsible for much of this advance.

The effect of these compounds on the practice of medicine itself has been more subtle. It appears that respect for the medical profession has deteriorated as the reliance on new drugs has increased. The change in the climate of medical practice is influenced by the natural social evolution with accumulation of greater wealth and increasing materialistic philosophy in both patients and physicians. Frequent moving about of family units with disruption of close ties with relatives and the family physician is part of the trend of our times. A profusion of popular articles on medicine and drugs and especially on toxic reactions to drugs causes uncertainty and a measure of distrust. In large part, however, this change is due to loss of fear of disease and especially of acute illness, because of the antibiotic drugs. Fear is one of the driving forces that brings many patients to the physician's office.

This becomes apparent with each new cancer crusade or poliomyelitis epidemic, or when an important or newsworthy person becomes ill or dies. Misguided dependence on drugs has affected physicians as well, leading to inertia of thought and action and overuse of the prescription form. Nowhere is this more evident than in our present difficulties with hospital staphylococci. Physicians have fostered this attitude by frequently phoning prescriptions for antibiotic and other drugs without examination of the patient.

Moreover, with our eagerness to develop new and more potent drugs, we have frequently neglected research in the basic sciences. Particularly is this true in the fields of immunology and host resistance to disease.

A brief review of some advances in pharmacological treatment and their effect on medical practice appears in order. Antibiotics are the most commonly prescribed drug. The synthetic production of penicillin should represent a major breakthrough in chemotherapeutics. Improved oral tetracycline derivatives have shown higher and longer-sustained effective blood levels. Griseofulvin, a peculiar antibiotic in that it is absorbed systemically and deposited in the keratin of skin, hair, and nails, inactivates all species of dermatophytic fungi. Although prolonged treatment has been necessary in many fungus infections the results have been excellent. An oral nitrofurantoin preparation is available, though its future is far from secure. Sulfonamides have not been neglected, and several new compounds have been introduced that are effective in one or two daily doses. This type of agent would appear to be superior for the prophylaxis of streptococcal infections, but to date there are no supporting data.

Some years ago, in a book entitled *Psychosocial Medicine*, Haliday depicted the psychosomatic difficulties that every recorded civilization went through with its social evolution. His predictions for the United States have been adequately borne out, and it is no surprise that considerable attention has been focused on the psychopharmaceutical drugs. Aldous Huxley has noted that there is nothing new about the concept of ataractic drugs, and he defined them as affecting the quality of consciousness. It should be noted in passing that barbiturate manufacture has increased 1000 per cent since 1933, and the annual consumption is now estimated at 300 tons. Our chief concern, however, is with the so-called ataraxics. With the advent of these compounds, a profound change in the treatment of psychotic patients was effected. The necessity for cold tubs and electroshock therapy was greatly reduced. The mental hospitals were able to discharge or furlough many patients and others were made more amenable to psychotherapy. Many severe psychoneurotic symptoms were also brought under control, allowing the family physician an opportunity to help the overworked psychiatrist in treating many of these problems.

Ataractic drugs may be divided into two groups, those that affect the autonomic nervous system, such as the *Rauwolfia* alkaloids and the phenothiazine derivatives, and the central relaxants, such as meprobamate. These groups differ markedly from one another in method of action and side reactions. They are probably frequently prescribed without this knowledge.

A more recent introduction are the hydrazine compounds, which affect the enzyme amine oxidase. These drugs inhibit the breakdown of the biogenic amines such as serotonin, norepinephrine, and epinephrine. Encouraging results have been reported from clinical use in depression and angina pectoris. An interesting use of these compounds has been in the isolation of an active phenylalanine metabolite, which appears to be produced in excess in phenylpyruvic oligophrenia. The enzymatic destruction of this substance is blocked, and measurable amounts are excreted in the urine.

The studies of use of iproniazid in angina pectoris were encouraging, though the drug was too toxic for continued use. These studies are being repeated with less toxic derivatives and other related compounds. The bombardment with advertising for the amine oxidase inhibitors in the last months has been heavy. It appears to be very premature and these compounds may possibly lead to harmful results. The treatment of depression calls for a definitive diagnosis that may be beyond the capacity of the general physician. The dosage of these drugs has not been clearly established and a period of time must elapse before results can be expected. Such advertising methods suggest the need for more regulation over the release of some new drugs. It appears that if the pharmaceutical manufacturers or the physicians cannot control this situation effectively, such a regulation will be effected by outside agencies.

In this same field, the effects of extracts of bovine pineal gland substances in schizophrenic patients and the production of schizophrenic-like symptoms in man after the administration of certain drugs suggest the eventual use of enzyme inhibitors or enzyme protectors in the treatment of this disease.

Interest in the metabolic problems of diabetes mellitus has been rekindled by the studies of Loubatieres and others on the effect of oral agents in lowering blood sugar. As a result of this work, two sulfonylurea agents (tolbutamide, chlorpropa-

mide) are now in general use in treating mild diabetes of older people. A third drug in the biguanidine series (phenformin) has also been introduced. A tremendous literature has resulted from the use of these agents and many persons with mild diabetes have been helped. It seems safe to predict that these drugs will contribute more to the fundamental understanding of diabetes mellitus than to its actual treatment and that they will be replaced by more active and less toxic agents.

The search for antipressor drugs for the treatment of hypertension continues apace. The latest agents to be reported are some of the amine oxidase inhibitors. There is no single compound that has a widespread, profound clinical effect in lowering high blood pressure. Combinations of veratrum alkaloids, *Rauwolfia* alkaloids, hydralazine, ganglionic blocking agents, and hydrochlorothiazide have been widely successful for the treatment of essential hypertension and some hypertensive crises, such as in toxemia of pregnancy. The *Rauwolfia* alkaloids have been additionally helpful because of a sedative and cardiac decelerator action. The mode of action and toxicity of these compounds are exceedingly complex, so that we merely mention them at this time.

The relation of cholesterol to hypertension and atherosclerosis is confusing, but the results of Mer 29 in lowering serum cholesterol and relieving angina pectoris are quite promising. This compound apparently interferes with cholesterol synthesis and has a low order of toxicity.

Studies with diuretic drugs have progressed from xanthines to mercury compounds to carbonic anhydrase inhibitors to chlorothiazide and related compounds and finally to aldosterone antagonists. The diuretic and saluretic effects of chlorothiazide, hydrochlorothiazide, and hydroflumethiazide are great, but also potentially toxic because of potassium loss. Again we have been bombarded with a rash of claims and counterclaims regarding the efficacy and relative toxicity of these compounds. The results of animal experiments have been freely translated to human beings.

Some of these compounds are so new that they are supported almost entirely by personal communications. In effect there is no way for a physician to judge the accuracy of the claims. There is also a recent tendency to add potassium to these compounds to counteract its loss. This could well result in disaster in some patients receiving digitalis or with kidney disease. The additional possibility of nephropathic lesions resulting from chronic hypokalemia has not been well appreciated.

Streptokinase-streptodornase, trypsin, pancreatic dornase, and human plasmin have been used to lyse both intra- and extravascular clots, and for the débridement of wounds and abscess cavities and the liquefaction of gelatinous tracheobronchial secretions. The results, especially in thrombophlebitis and pulmonary embolism, have been varying but encouraging. The advantage of removing clots in cerebrovascular, coronary artery, and pulmonary artery disease is obvious. A number of side reactions, especially chills and fever, have been reported with the use of human plasmin. Newer compounds are now being tried, apparently with fewer side reactions.

Evaluation of steroids involves problems in that they are used in a great variety of conditions of uncertain etiology and unpredictable courses. The development of more potent compounds with lowering of dosage has resulted in fewer side reactions. Their use in various allergic and endocrine disorders and some usually

fatal diseases such as pemphigus has been lifesaving. However, the aspirations of many at the time of introduction of these compounds have not been fulfilled.

On the other hand, the antihistamine drugs have a more definitive application and have been of great value. We are plagued with such a plethora of compounds, however, that we may ask, "how much is enough?" The introduction of most of these drugs reflects little credit on the manufacturers.

Diethylpropion, a new anorexigenic compound that curbs appetite without undesirable stimulation of the central nervous system, may replace the amphetamines for this purpose.

Imferon, a superior intramuscular preparation of iron, has been effective, though it produces more side reactions than we have been led to believe.

In passing, it would be well to mention that we could use some effective drugs for such common problems as the relief of pain, itching, diarrhea, and cough.

Despite all these dramatic improvements in the diagnosis and treatment of many diseases, recent biochemical, genetic, and enzymatic studies portend an even greater era of medicine in which the problems of mental disease, atherosclerosis, diseases due to inborn metabolic errors, and perhaps even leukemia and cancer will be overcome. We must not stop to congratulate ourselves that we have overcome certain diseases, since there are others yet to be conquered.

As more potent and potentially more toxic drugs are introduced, together with the improvement in diagnosis and medical practice that they bring about, the difficulty of bringing this knowledge to the attention of our practicing physicians becomes more acute. The Aug. 22, 1959, issue of the *Journal of the American Medical Association* among other articles included the following: "Human Pharmacology of Thiazide Derivatives," "Acute Pancreatitis in Patients Receiving Chlorothiazide," "Clinical Therapeutic Evaluation of Hydrochlorothiazide (Hydrodiuril)," "Hemorrhage from Multiple Sites Associated with Chlorpromazine-Induced Jaundice," "Ototoxicity of Kanamycin," "Agranulocytosis Caused by Phenothiazine Derivatives," "Hypokalemic Muscle Paralysis Associated with Administration of Chlorothiazide," "Hepatic Damage during Chlorpropamide Therapy." Here is a clear illustration of our problem, and the list of chemical compounds also emphasizes the importance of including trade names parenthetically.

The conscientious use of newer drugs demands an understanding of advances in physiology and pharmacology. Without this knowledge the proper selection of drugs cannot be made. This raises the entire problem of postgraduate education. The various specialty groups and, more recently, the Academy of General Practice have encouraged such education, but their efforts lack direction. Much more needs to be done. A "Flexner type" of survey, preferably under the auspices of the American Medical Association, would show our needs and help in planning to meet them. The American Medical Association itself should be greatly strengthened so that membership would be more meaningful to the public. Let membership imply excellence in the ethical practice of medicine. Study courses should be set up utilizing teams of clinicians and research workers from the universities and the drug industry under the direction of local, county, and state medical societies. In this way the needs of local physicians could be best interpreted and met. If necessary, attendance at these courses should be compulsory and they should be open also to drug detail men and medical science writers. It is obvious that unless our rapid

improvement in diagnosis and therapy is translated into action by our general physicians, little good from it will come to the public.

To return specifically to drug therapy, there are several aspects of our present method of disseminating drug information that should be discussed. The successful busy practitioner must learn to budget his time, and in such a schedule there is little room for haphazard reading. The great majority of advertising material and pharmaceutical pamphlets and bulletins, though skillfully conceived, fit into this category. The deluge of material received prohibits even a casual examination. This problem is recognized and a change in format in many bulletins has occurred. There is a skillful interweaving of socioeconomic reports, advertising, and reports on advances in medicine. Good examples are the news reports sponsored by The Upjohn and Ciba Pharmaceutical Companies, for which they are to be commended.

Similar problems arise in discussion with detail representatives. For the most part, their visits occur during the busy office schedule when the physician is fatigued and harassed by telephone calls and problem cases. Many of these detail men are well trained and understand the basic research, but they are indoctrinated with the company line. They do not know how their products compare with those already in use. They do not know the utter worthlessness of many reprints that are used in promoting their products. Many of these articles are uncritical, uncontrolled, and poorly conceived. Unfortunately many physicians lack the training to recognize these same inadequacies.

We may obtain information on minimal lethal dose in mice, the effect on the guinea pig gut or the rabbit uterus, but only rarely clinical comparisons in human beings.

Some of these problems could be met by more dependence on symposia of the type sponsored by the New York Academy of Sciences as a basis for detail of products.

Increasing difficulties are arising through the premature release of drug information to the press. This has resulted in a public demand for a product before the practicing physician has an opportunity to study the compound. Such a practice is properly condemned.

The Food and Drug Administration has done an outstanding job. The Council on Drugs of the American Medical Association could do much more if its scope of activity were widened. Yearly comparative reviews of basic drugs should be published. A subcommittee of the Council should review as many popular medical writings as possible. Those that are flagrantly misleading or taken out of context should be widely exposed. *Today's Health* magazine should be more widely circulated to give the public more factual information on advances in medicine and drug therapy. Greater use should be made of closed-circuit television with panel discussions on drug therapy.

While the pharmaceutical manufacturer has a serious obligation in releasing new drugs, let us recognize that the final responsibility clearly rests on the prescribing physician. This responsibility can be discharged only when the physician is thoroughly acquainted with the condition to be treated and the pharmacology and toxicology of the agent to be prescribed.

Despite all adjuvants in the form of advertising or articles, this need can only be filled by postgraduate study courses.

Microbiological and Pharmacological Studies of Colistin Sulfate and Sodium Colistinmethanesulfonate

BENJAMIN S. SCHWARTZ, MARSHALL R. WARREN, FRED A. BARKLEY, AND
LARRY LANDIS

Warner-Lambert Research Institute, Morris Plains, N. J.

A new antibiotic that has been given the generic name colistin was described by Koyama and his associates¹ in 1950. This antibiotic was obtained from *Bacillus (Aerobacillus) colistinus*, a new species, isolated from a soil sample from Fukushima Prefecture, Japan. Two forms are commercially available: colistin sulfate (Coly-mycin S*) and sodium colistinmethanesulfonate (Coly-mycin M†). Both of these have been studied and used for several years in Japan,^{2 12} Italy,^{13 35} and France,³⁶⁻⁴⁰ and for the past year in this country.

Colistin sulfate and sodium colistinmethanesulfonate possess marked bacteriostatic and bactericidal activity in vitro against a wide variety of gram-negative bacteria and lesser activity against gram-positive bacteria and fungi. In vitro studies indicate that there is no appreciable reduction in activity in the presence of serum. The in vitro development of resistant strains from those naturally sensitive to these antibiotics is not readily accomplished. Furthermore, most of the resistant strains thus isolated do not prove to be stable and readily revert in the absence of the antibiotic. These observations are in agreement with those reported by Koyama et al.,¹ Moiraghi-Ruggenini,⁴¹ and Chabbert.³⁸ There is no evidence of cross resistance to the presently known broad-spectrum antibiotics, and resistance incident to therapy has not been reported. High chemotherapeutic activity and a favorable therapeutic index can be demonstrated for both forms using several experimentally induced gram-negative infections in mice.

Chemically, colistin sulfate and sodium colistinmethanesulfonate are polypeptides; however, they differ chemically and pharmacologically from any of the known polypeptide antibiotics. In man, single intramuscular therapeutic doses of sodium colistinmethanesulfonate result in high blood serum levels for a period of 8 to 12 hours, with detectable levels up to 24 hours. This antibiotic is excreted fairly rapidly in the urine, as evidenced by high urine concentrations and total excretion over a 24 hour period. Colistin sulfate and sodium colistinmethanesulfonate are not readily absorbed from the gastrointestinal tract. Subacute toxicity studies in rats and dogs indicate that these antibiotics are not nephrotoxic. Clinical studies in this country and clinical experience for several years in Japan, Italy, and France have borne this out.

Many investigators have studied the impact of intensive usage of the broad-spectrum antibiotics (streptomycin, the tetracyclines, and chloramphenicol) on urinary tract and other infections due to *Proteus*, *Pseudomonas*, and coliform

* The trade name of Warner-Chilcott Laboratories for colistin sulfate is Coly-mycin S.

† The trade name of Warner-Chilcott Laboratories for sodium colistinmethanesulfonate is Coly-mycin M.

organisms.⁴²⁻⁴⁴ In general, such studies have disclosed steady increases in the incidence of antibiotic-resistant strains. The relatively more toxic antibiotics (polymyxin, neomycin, and bacitracin) have been successfully employed in the treatment of infections due to gram-negative bacilli, especially those resistant to the broad-spectrum antibiotics.⁴⁵ Nevertheless, there remains a great need for a nontoxic, highly bactericidal agent for more general use in such infections, preferably one to which these organisms are unable to become resistant. Our studies and the clinical experience in this country indicate that colistin sulfate and sodium colistimethanesulfonate may well fill an important chemotherapeutic need in this area.

MATERIAL AND METHODS

Agents. The samples of colistin sulfate and sodium colistimethanesulfonate used in these studies were prepared and supplied by Kayaku Antibiotic Research Company, Ltd., Tokyo, Japan. Two lots of colistin sulfate, with potencies of 18,000 and 20,000 units/mg., respectively, and a single lot of sodium colistimethanesulfonate, with a potency of 11,500 units/mg., were used. Pure colistin base has been assigned a potency of 30,000 units/mg. (30 units \approx 1 μ g.).

For the microbiological studies, solutions were prepared in the following manner. Sterile aqueous stock solutions of colistin sulfate and sodium colistimethanesulfonate were prepared in terms of base equivalents, and potencies and activities are expressed in terms of colistin base. Commercially available preparations of the other antibiotics were employed. Polymyxin B sulfate* was obtained in a sterile powder, 500,000 units equivalent to 50 mg. polymyxin standard. Since its potency was expressed on a unitage and equivalence basis, each vial was reconstituted with sterile distilled water to give a stock solution of 5000 μ g./ml. in terms of polymyxin standard. Vials of streptomycin sulfate, containing the equivalent of 10 Gm. of pure streptomycin base, were reconstituted with sterile distilled water to give a stock solution of 1,000,000 μ g./ml. Sterile aqueous stock solutions of penicillin G were prepared using vials of crystalline potassium penicillin G containing 5,000,000 units/vial, with a potency of 1580 units/mg. The sterile stock solutions of each of these antibiotics were ordinarily used on the same day, and never after three days' storage in the refrigerator.

In Vitro Tests. The organisms tested were obtained either from the American Type Culture Collection (ATCC), from our own stock culture collection, or from recent isolates from patients. The two latter are all designated as WLRI, and those strains that are resistant to one or more antibiotics are followed by the letter "R."

Two basic media were used in the in vitro studies: tryptose phosphate broth (Difco) for bacteria, and Sabouraud's liquid medium (Difco) for fungi. In the case of some fastidious bacteria, the basic medium was fortified with 1 per cent horse serum. The determination of the minimal inhibitory concentration was carried out by the usual twofold or threefold serial dilution method. For bacteria and *Candida* strains, 1 ml. of a 1:1000 dilution of an 18 hour tryptose phosphate broth culture was used as the inoculum. With the filamentous fungi, the inoculum was a 3 mm. plug from a 10 to 15 day old culture of the organism on Sabouraud's dex-

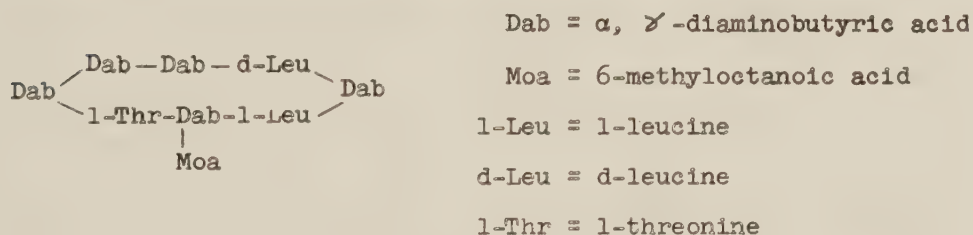
* The trade name of Burroughs Wellcome & Co. for polymyxin B sulfate is Aerosporin sulfate.

trose agar (Difco). The bacterial cultures were incubated for 48 hours at 37 C. and the fungal cultures for 72 hours at 20 C. The minimal inhibitory concentration was recorded for each organism as the lowest concentration of the compound at which there was no visible turbidity.

Therapeutic Tests. Female albino mice (Barchmann Farms, strain IS-32), weighing 18 to 22 Gm., were used. Two organisms, received from R. J. Schnitzer, were used in the therapeutic comparisons: *Escherichia coli* "J" and *Klebsiella pneumoniae* 1. Cultures were prepared by mouse passage the day before they were to be used, and heart blood transfers were grown in blood broth for 18 hours at 37 C. The mice were infected by the intraperitoneal injection of 0.5 ml. of culture. For *E. coli*, a 1:100 dilution in 5 per cent gastric mucin was used; for *K. pneumoniae*, a 1:1000 dilution in saline. These amounts resulted in inocula averaging 870,000 bacteria in the case of *E. coli*, and 82,000 in that of *K. pneumoniae*. The drug dilutions were administered in 0.5 ml. volumes. The mice were treated within one hour after infection; when multiple treatments were employed, additional treatments were given at 7, 23, and 29 hours after the infecting dose. All mice were observed for 10 days. Additional pertinent information in regard to materials and methods will be presented in the appropriate sections.

CHEMISTRY

Colistin is a white basic polypeptide whose salts are soluble in water and are



or

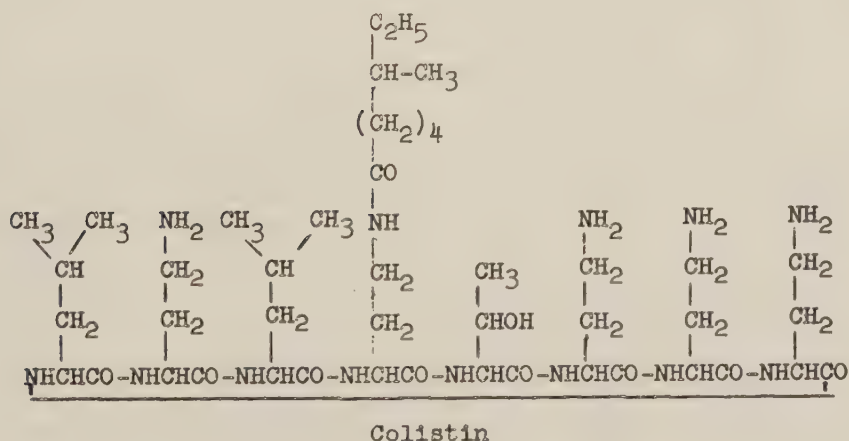
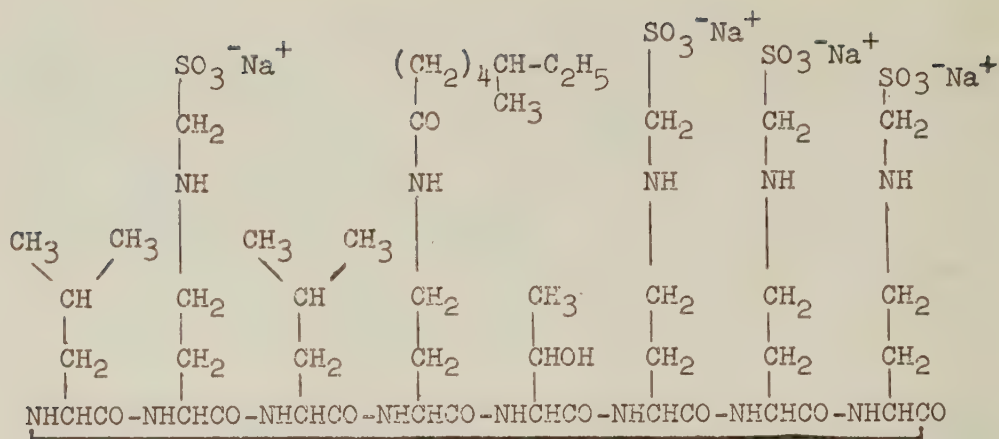


FIG. 1. Proposed structure of colistin.



Sodium colistinmethanesulfonate

FIG. 2. Proposed structure of sodium colistinmethanesulfonate.

levorotatory. The base has a molecular weight of approximately 969 and the empirical formula $C_{45}H_{85}O_{10}N_{13}$.⁵ Analyses of the acid hydrolysate of colistin show it to be composed of five moles of α , γ -diaminobutyric acid and one mole each of 6-methyloctanoic acid, 1-threonine, 1-leucine, and d-leucine.⁵ End group analyses by Sanger's method show the basicity of colistin to be due to the free γ -amino groups of four moles of α , γ -diaminobutyric acid.⁴ The other functional group found to be present is the free hydroxyl group of 1-threonine. Partial hydrolysate analyses of colistin coupled with these findings have led to the proposed structure shown in figure 1.

Solutions of colistin salts are relatively stable at pH 2 to 6 but decrease in stability above pH 6. The salts in the solid form are quite stable even at elevated temperatures.

Paper chromatography of colistin by several workers has served to show its non-identity with related antibiotics of the cyclic polypeptide types.^{4, 46-48}

Sodium colistinmethanesulfonate can be considered to be a methylated form of colistin and is prepared by treating colistin with formaldehyde and sodium bisulfite. Its structure is represented as shown in figure 2.

IN VITRO ANTIMICROBIAL ACTIVITY

In Vitro Spectra. The in vitro spectra of colistin sulfate, sodium colistinmethanesulfonate, polymyxin B sulfate, and streptomycin sulfate were determined with 112 strains representing 16 genera of bacteria and 27 strains representing 12 genera of fungi. In table I are shown the minimal inhibitory concentrations for bacterial strains. Repeated tests, using the same strains, showed slight but not significant variations in these concentrations. The intrinsic activity of colistin sulfate for these strains resembled that of polymyxin B sulfate; however, there were a few exceptions. Sodium colistinmethanesulfonate, for the most part, was slightly less active. With the exceptions of *Proteus* and *Neisseria* in the gram-negative strains, colistin sulfate, polymyxin B sulfate, and sodium colistinmethanesulfonate were generally more effective than streptomycin. The data also showed that streptomycin-resistant strains

TABLE I

Comparative Antibacterial in Vitro Spectra of Colistin Sulfate, Sodium Colistinmethanesulfonate, Polymyxin B Sulfate, and Streptomycin Sulfate

			Minimal inhibitory concentration, µg./ml.			
Organism			Colistin sulfate	Sodium colistin-methanesulfonate	Polymyxin B sulfate	Streptomycin sulfate
Gram Negative						
<i>Aerobacter aerogenes</i>	WLRI	55R	1.23	>33.3	0.41	4.1
	WLRI	108	0.14	0.41	0.02	4.1
	WLRI	109	0.02	0.41	0.02	12.3
	WLRI	143R	>100.0	>100.0	>100.0	>3000.0
	WLRI	144	11.1	11.1	3.7	4.1
	WLRI	200	33.3	33.3	11.1	12.3
<i>Bordetella bronchiseptica</i>	ATCC	9617	33.3	>33.3	>100.0	1000.0
	WLRI	221	0.01	0.01	0.01	12.3
	WLRI	211	3.7	3.7	11.1	333.0
<i>Brucella abortus</i>	WLRI	81	>100.0	>100.0	>100.0	1.3
<i>Escherichia coli</i>	WLRI	51R	0.01	0.04	0.02	12.3
	ATCC	6883	0.01	0.14	0.14	>3000.0
	WLRI	53	0.02	0.04	0.02	12.3
	WLRI	105	0.01	0.41	>100.0	12.3
	WLRI	126R	0.02	0.14	0.02	333.0
	WLRI	127R	0.02	1.2	0.02	12.3
	WLRI	128R	>100.0	>100.0	>100.0	333.0
	WLRI	130	0.01	0.41	0.02	4.1
	WLRI	134R	0.01	0.04	0.02	>3000.0
	WLRI	131R	0.01	0.14	0.02	4.1
	"B"	WLRI 132R	3.7	3.7	11.1	1000.0
		WLRI 135R	0.41	0.41	0.12	37.0
		WLRI 136	0.01	0.41	3.7	12.3
		WLRI 137R	>100.0	>100.0	>100.0	111.0
		WLRI 138R	0.01	0.14	11.1	1.3
		WLRI 140	0.04	0.14	0.14	12.3
		WLRI 159R	0.01	0.14	0.02	>3000.0
	"J"	WLRI 206	0.01	0.14	0.02	4.1
		WLRI 220	0.01	0.14	0.02	333.0
<i>Klebsiella pneumoniae</i>	WLRI	217	0.01	0.14	0.05	0.45
	WLRI	45	1.2	3.7	0.41	4.1
	WLRI	218	0.02	0.01	0.02	4.1
	WLRI	219	0.02	0.14	0.02	12.3
	WLRI	203	0.01	0.41	0.02	1.3
<i>Neisseria meningitidis</i>	WLRI	71	3.7	>33.3	>100.0	0.45
	WLRI	72	33.3	>33.3	>100.0	4.2
<i>Neisseria catarrhalis</i>	WLRI	75	>100.0	>100.0	>100.0	0.45
<i>Paracolobactrum coliforme</i>	WLRI	112	>100.0	>100.0	>100.0	>3000.0
<i>Proteus morgani</i>	WLRI	160	>100.0	>100.0	>100.0	>3000.0
	WLRI	163	>100.0	>100.0	>100.0	111.0
<i>Proteus species</i>	WLRI	56	>100.0	>100.0	>100.0	111.0
	WLRI	58	>100.0	>100.0	>100.0	>3000.0
	WLRI	57	>100.0	>100.0	>100.0	>3000.0
	WLRI	59	>100.0	>100.0	>100.0	>3000.0
	WLRI	60	>100.0	>100.0	>100.0	>3000.0
	WLRI	83	>100.0	>100.0	>100.0	111.0
	WLRI	84	>100.0	>100.0	>100.0	111.0
	WLRI	164R	>300.0	>300.0	>300.0	111.0

Table I Continued on Page 46

TABLE I (Continued)

Comparative Antibacterial in Vitro Spectra of Colistin Sulfate, Sodium Colistinmethanesulfonate, Polymyxin B Sulfate, and Streptomycin Sulfate

Organism			Minimal inhibitory concentration, µg./ml.			
			Colistin sulfate	Sodium colistin-methane-sulfonate	Polymyxin B sulfate	Streptomycin sulfate
Gram Negative (Continued)						
<i>Proteus vulgaris</i>	WLRI 204	>100.0	>100.0	>100.0	111.0	
	ATCC 93	>100.0	>100.0	>100.0	37.0	
	WLRI 191	>100.0	>100.0	>100.0	111.0	
	ATCC 881	>300.0	>300.0	>300.0	12.3	
	WLRI 139R	>100.0	>100.0	>100.0	111.0	
<i>Pseudomonas aeruginosa</i>	ATCC 9027	0.14	1.2	0.02	37.0	
	WLRI 216	1.2	11.1	0.41	>3000.0	
	WLRI 202	3.7	33.3	3.7	37.0	
<i>Pseudomonas dentrificans</i>	WLRI 63	0.02	0.14	0.01	>1000.0	
<i>Pseudomonas fluorescens</i>	WLRI 100	33.3	33.3	11.1	4.1	
	WLRI 208	3.7	33.3	3.7	111.0	
	WLRI 47	0.04	0.43	0.14	333.0	
<i>Salmonella choleraesuis</i>	WLRI 50	0.04	0.04	0.02	111.0	
	WLRI 101	0.01	0.41	0.02	111.0	
<i>Salmonella enteriditis</i>	WLRI 91	0.01	0.41	0.02	333.0	
	WLRI 141	0.04	0.41	0.02	37.0	
<i>Salmonella gallinarum</i>	WLRI 129	0.02	0.14	0.02	333.0	
<i>Salmonella paratyphi</i> "A"	WLRI 86	3.7	3.7	0.41	333.0	
	WLRI 87	0.02	0.04	0.02	12.3	
<i>Salmonella schottmuelleri</i> "B"	WLRI 193	0.02	0.14	0.05	37.0	
	WLRI 88	0.01	0.14	0.02	37.0	
	WLRI 205	0.02	0.41	0.02	111.0	
<i>Salmonella species</i>	WLRI 49	0.01	0.41	0.02	100.0	
	WLRI 106	3.7	3.7	1.23	111.0	
<i>Salmonella typhimurium</i>	WLRI 142	0.41	0.41	0.02	111.0	
<i>Salmonella typhosa</i>	WLRI 48	0.01	0.14	0.02	111.0	
	WLRI 207	0.01	0.14	0.02	37.0	
<i>Serratia marcescens</i>	ATCC 6889	11.1	11.1	11.1	0.45	
<i>Serratia species</i>	WLRI 78	>300.0	>300.0	>300.0	1000.0	
<i>Shigella dysenteriae</i>	WLRI 94	0.01	0.14	0.02	12.3	
	WLRI 98	0.01	0.01	0.02	12.3	
<i>Shigella paradysenteriae</i>	WLRI 97	0.01	0.14	0.01	111.0	
	WLRI 201	0.01	0.14	0.02	12.3	
<i>Shigella sonnei</i>	ATCC 4950	0.01	0.14	0.02	12.3	
	WLRI 46	0.01	0.14	0.02	37.0	
Gram Positive						
<i>Bacillus cereus</i>	ATCC 9634	11.1	>33.0	100.0	4.1	
<i>Bacillus megatherium</i>	WLRI 62	300.0	300.0	111.1	0.41	
	WLRI 42	11.1	0.4	0.5	11.1	

Table I Continued on Page 47

TABLE I (Continued)

Comparative Antibacterial in Vitro Spectra of Colistin Sulfate, Sodium Colistinmethanesulfonate, Polymyxin B Sulfate, and Streptomycin Sulfate

Organism			Minimal inhibitory concentration, µg./ml.			
			Colistin sulfate	Sodium colistin-methanesulfonate	Polymyxin B sulfate	Streptomycin sulfate
Gram Positive (Continued)						
<i>Bacillus subtilis</i> var. <i>niger</i>	WLRI	43	33.0	11.1	3.7	111.0
<i>Corynebacterium diphtheriae</i>	WLRI	96	0.4	1.2	0.14	0.14
<i>Sarcina lutea</i>	WLRI	209	11.1	33.3	33.3	4.1
<i>Staphylococcus aureus</i>	WLRI	19R	>100.0	>100.0	>300.0	333.0
	WLRI	20R	100.0	100.0	11.1	>300.0
	WLRI	24	33.0	33.0	11.1	4.1
	WLRI	25	11.1	11.1	1.23	1.3
	WLRI	180R	100.0	100.0	100.0	1.3
	WLRI	181R	100.0	100.0	100.0	1.3
	WLRI	182R	100.0	100.0	100.0	100.0
	WLRI	183R	100.0	100.0	100.0	>300.0
	WLRI	184R	100.0	100.0	100.0	1.3
	WLRI	185R	100.0	100.0	100.0	100.0
	WLRI	186R	100.0	33.0	11.1	100.0
	WLRI	187R	100.0	100.0	100.0	100.0
	WLRI	188R	100.0	100.0	100.0	100.0
	WLRI	189R	100.0	100.0	100.0	100.0
<i>Streptococcus faecalis</i>	WLRI	41R	>100.0	>100.0	>300.0	9000.0
	WLRI	40	>100.0	>100.0	>300.0	111.0
	ATCC	8022	>100.0	>100.0	>100.0	—
	ATCC	6569	>100.0	>100.0	>100.0	—
	ATCC	7080	>100.0	>100.0	>100.0	—
<i>Streptococcus pyogenes</i>	WLRI	38	33.0	33.0	11.1	12.3
<i>Streptococcus viridans</i>	ATCC	9758	>100.0	>100.0	>100.0	—
	ATCC	9222	33.0	33.0	33.0	—
	ATCC	9756	>100.0	>100.0	>100.0	—

of gram-negative organisms may be sensitive to the other three antibiotics. The *Proteus* and *Neisseria* strains generally resistant to colistin sulfate, sodium colistinmethanesulfonate, and polymyxin B sulfate were in several instances moderately responsive to streptomycin. Most *Pseudomonas* strains sensitive to the three antibiotics required high concentrations of streptomycin for inhibition. All four antibiotics were generally less active against the gram-positive cocci and in most instances exhibited only limited activity. Against the gram-positive bacilli, streptomycin was the most active of the four. Essentially the same observations have been reported for colistin by Koyama,^{1,5} Chabbert,³⁸ Moiraghi-Ruggenini,⁴⁹ Ito et al,⁵⁰ Osawa and Nakamura,⁵¹ and for the polymyxins and streptomycin by Bliss and Todd,⁵² Kagan et al,⁵³ Pulaski et al,⁵⁴ Frank et al,⁵⁵ and Wright et al.⁵⁶

The relative activity against representative fungi strains is shown in table II. Colistin sulfate, sodium colistinmethanesulfonate, and polymyxin B showed moderate activity principally against several *Candida* strains, whereas streptomycin was

TABLE II

Comparative Antifungal in Vitro Spectra of Colistin Sulfate, Sodium Colistinmethanesulfonate, Polymyxin B Sulfate, and Streptomycin Sulfate

			Minimal inhibitory concentration, $\mu\text{g./ml.}$			
Organism			Colistin sulfate	Sodium colistin-methanesulfonate	Polymyxin B sulfate	Streptomycin sulfate
<i>Aspergillus flavus</i>	WLRI	300F	>100.0	>100.0	300.0	>9000.0
<i>Aspergillus fumigatis</i>	ATCC	1022	>100.0	>100.0	>300.0	>9000.0
<i>Aspergillus niger</i>	ATCC	1044	>100.0	>100.0	>300.0	>9000.0
<i>Blastomyces dermatidis</i>	WLRI	305F	>100.0	>100.0	>300.0	>9000.0
<i>Blastomyces allii</i>	WLRI	304F	3.7	100.0	11.1	>9000.0
<i>Candida albicans</i>	WLRI	329F	>100.0	>100.0	300.0	>9000.0
	WLRI	330F	>100.0	>100.0	33.3	>9000.0
	WLRI	331F	>100.0	>100.0	100.0	>9000.0
	ATCC	10231	>100.0	>100.0	300.0	>9000.0
	WLRI	337F	>100.0	>100.0	300.0	>9000.0
<i>Candida brumptii</i>	WLRI	340F	11.1	33.3	33.3	>9000.0
<i>Candida krusei</i>	ATCC	6258	33.3	100.0	11.1	>9000.0
<i>Candida flaveri</i>	WLRI	338F	1.3	3.7	.4	>9000.0
<i>Candida mycoderma</i>	ATCC	9888	11.1	100.0	11.1	>9000.0
<i>Candida pulcherrima</i>	WLRI	339F	33.0	33.0	11.1	>9000.0
<i>Candida tenuis</i>	WLRI	341F	33.0	>100.0	11.1	>9000.0
<i>Candida tropicalis</i>	ATCC	750	11.1	100.0	3.7	>9000.0
<i>Cryptococcus neoformans</i>	WLRI	309F	>100.0	>100.0	>300.0	>9000.0
<i>Epidermophyton floccosum</i>	WLRI	310F	>100.0	>100.0	300.0	>9000.0
<i>Microsporum canis</i>	ATCC	10214	>100.0	100.0	100.0	>9000.0
<i>Monilia fructicola</i>	WLRI	327F	11.1	33.3	11.1	>9000.0
<i>Penicillium digitatum</i>	ATCC	10030	>100.0	>100.0	>300.0	>9000.0
<i>Phomopsis citri</i>	ATCC	9054	33.3	>100.0	>300.0	>9000.0
<i>Sclerotinia sclerotiorum</i>	WLRI	326F	>100.0	>100.0	>300.0	>9000.0
<i>Sporotrichum schenkii</i>	ATCC	10213	>100.0	>100.0	>300.0	>9000.0
<i>Trichophyton mentagrophytes</i>	WLRI	320F	33.0	100.0	100.0	>9000.0
<i>Trichophyton violaceum</i>	WLRI	325F	>100.0	>100.0	>300.0	>9000.0

inactive. Similar activity was reported for the polymyxins by Simon⁵⁷ and Florestano and Bahler⁵⁸ and for colistin by Forni.²³

Effect of Size of Inoculum. Experience with the polymyxins^{52, 59, 60, 63} and colistin³⁸ showed that titration end points with these antibiotics, and in fact with most others, depended on the number of bacteria inoculated into the media. The influence of size of inoculum on in vitro activity of colistin sulfate and sodium colistinmethanesulfonate was studied, using *Pseudomonas aeruginosa* ATCC 9027 and *K. pneumoniae* WLRI 45 as the test organisms. The results are illustrated in table III. Colistin sulfate appeared less affected by size of inoculum than was sodium colistinmethanesulfonate.

The increase in the minimal inhibitory concentration was approximately tenfold,

TABLE III

Effect of Size of Inoculum on Titration End Points

Organism	No. of cells	Minimal inhibitory concentration, $\mu\text{g./ml.}$	
		Colistin sulfate	Sodium colistinmethanesulfonate
<i>Ps. aeruginosa</i> ATCC 9027	10^3	<0.03	0.03
	10^4	<0.03	0.06
	10^5	0.03	0.12
	10^6	0.12	0.5
	10^7	0.25	5.0
	10^8	0.50	10.0
<i>K. pneumoniae</i> WLRI 45	10^3	<0.13	<0.13
	10^4	0.13	0.13
	10^5	0.40	1.23
	10^6	1.23	3.72
	10^7	3.12	11.1
	10^8	3.12	33.3

Tryptose phosphate broth (Difco) incubated at 37 C. for 48 hours.

as the cell concentration increased from 10^3 to 10^7 . Cell concentrations higher than 10^7 resulted in greater reduction in activity. These results are not significantly different from those reported by Chabbert³⁸ for sodium colistinmethanesulfonate with *Shigella sonnei*.

Effect of pH. Although colistin sulfate and sodium colistinmethanesulfonate are stable for considerably longer periods at acidic than at alkaline pH, no significant differences in activity at pH 6, 7, and 8 were demonstrated. A wide variety of sensitive organisms in liquid media were tested at these pH values and incubated for 48 hours at 37 C. Chabbert³⁸ did, however, report that sodium colistinmethanesulfonate was 10 times more active at pH 6 than at pH 8 when evaluated in terms of zones of inhibition on *S. sonnei* seeded agar plates with discs impregnated with the antibiotic.

Effect of Serum. The results indicated that the presence of serum did not significantly influence the in vitro activity of colistin in liquid media. These results are

TABLE IV

Effect of Serum on in Vitro Antibacterial Activity

Microorganism	Minimal inhibitory concentration, $\mu\text{g./ml.}$					
	Colistin sulfate			Sodium colistinmethanesulfonate		
	T.P.*	T.P. plus 20% horse serum	T.P. plus 20% horse serum, 5% CO ₂	T.P.	T.P. plus 20% horse serum	T.P. plus 20% horse serum, 5% CO ₂
<i>E. coli</i> WLRI 51R	0.01	0.01	0.01	0.10	0.20	0.10
<i>S. paratyphi</i> WLRI 87	0.01	0.01	0.01	0.50	0.50	0.50
<i>S. sonnei</i> ATCC 4950	0.01	0.01	0.01	0.10	0.20	0.10
<i>A. aerogenes</i> WLRI 109	0.01	0.02	0.02	0.05	0.50	0.05

* T.P. = tryptose phosphate broth (Difco), with and without 20 per cent horse serum, incubated at 37 C. in air or 5 per cent carbon dioxide for 48 hours.

in agreement with those published by Chabbert³⁸ for sodium colistinmethanesulfonate, i.e., serum concentrations varying from 1 to 20 per cent gave only a two-fold increase in minimal inhibitory concentrations. Chabbert also claimed that any real difference in activity in the presence of serum was due to alkalization of the medium. He suggested that the tubes of such tests be incubated under 5 per cent carbon dioxide to prevent alkalization. In line with this suggestion, a number of tests were run under air and 5 per cent carbon dioxide incubation. A typical experiment is illustrated in table IV. Results with sodium colistinmethanesulfonate and *Aerobacter aerogenes* lend support to Chabbert's claim.

Bactericidal Activity. That the antibacterial action of colistin is primarily bactericidal and rapid has been repeatedly demonstrated here and by Chabbert³⁸ and others. The bactericidal activity can be readily demonstrated in the presence or absence of serum simply by subculturing from tubes that show no growth in the broth dilution tests. The bacteriostatic and bactericidal concentrations, for sensitive organisms in most cases, were not appreciably different. Essentially the same type of activity has been reported for the polymyxins.^{61 63}

TABLE V

Relative Activity of Polymyxin B Sulfate, Colistin Sulfate, and Sodium Colistinmethanesulfonate in Experimental Bacterial Infections in Mice (Four Subcutaneous Injections at 1, 7, 23, and 29 Hours Postinfection)

Antibiotic	<i>K. pneumoniae</i> 1				<i>E. coli</i> "J"	
	10 days postinfection				10 days postinfection	
	Dose, mg./Kg.	Alive/total	% effect	Dose, mg./Kg.	Alive/total	% effect
Polymyxin B sulfate	0.25	0/10	0	1.25	0/10	0
	0.5	0/10	0	2.5	0/10	0
	1.0	2/10	20.0	5.0	2/10	20.0
	2.0	7/10	70.0	7.5	3/10	30.0
	4.0	8/10	80.0	10.0	8/10	80.0
	6.0	10/10	100.0	12.5	8/10	80.0
Colistin sulfate	0.25	1/10	10.0	1.25	2/10	20.0
	0.5	2/10	20.0	2.5	5/10	50.0
	1.0	4/10	40.0	5.0	8/10	80.0
	2.0	5/10	50.0	7.5	7/10	70.0
	4.0	9/10	90.0	10.0	4/10	40.0 (toxic)
	6.0	8/10	80.0	12.5	2/10	30.0 (toxic)
Sodium colistin-methane-sulfonate	0.25	1/10	10.0	1.25	0/10	0
	0.5	4/10	40.0	2.5	1/10	10.0
	1.0	8/10	80.0	5.0	2/10	20.0
	2.0	8/10	80.0	7.5	5/10	50.0
	4.0	10/10	100.0	10.0	8/10	80.0
	6.0	9/10	90.0	12.5	8/10	80.0
Infected untreated controls		0/20			2/10	

TABLE VI

Relative Activity of Colistin Sulfate, Sodium Colistinmethanesulfonate, and Polymyxin B Sulfate in K. pneumoniae 1 Infections in Mice (Summary)

Antibiotic	Median effective dose,* mg./Kg.	Slope function†	Relative activity‡
<i>Four Subcutaneous Dosage Schedule</i>			
Polymyxin B sulfate	1.7 (1.07-2.70)	2.10 (1.81-2.41)	1.00
	1.6 (1.20-2.13)	1.60 (1.36-1.87)	1.06
Colistin sulfate	1.75 (1.09-2.80)	2.58 (1.51-4.37)	0.97
	1.49 (0.96-2.31)	2.45 (1.92-3.11)	1.14
Sodium colistinmethanesulfonate	0.78 (0.62-0.98)	3.23 (1.61-6.46)	2.17
	0.41 (0.24-0.68)	2.38 (1.24-4.56)	4.14
<i>Single Subcutaneous Dosage Schedule</i>			
Polymyxin B sulfate	2.5 (1.47-4.25)	2.30 (1.15-4.59)	1.00
Colistin sulfate	2.75 (1.66-4.44)	1.79 (1.14-2.79)	0.90
Sodium colistinmethanesulfonate	1.2 (0.57-2.52)	2.37 (1.49-3.75)	2.08
<i>Four Oral Dosage Schedule</i>			
Polymyxin B sulfate	24.0 (15.0-38.4)	2.13 (1.47-3.09)	1.00
Colistin sulfate	11.5 (7.03-18.6)	2.21 (1.38-3.54)	2.00
Sodium colistinmethanesulfonate	19.5 (12.8-29.64)	1.90 (1.33-2.28)	1.23

* Figures in parentheses are 95 per cent confidence limits.

† Slope function = $ED_{84}/ED_{80} + ED_{80}/ED_{16}$.

‡ Relative activity = ED_{80} of polymyxin B sulfate/ ED_{80} of the test antibiotic.

IN VIVO ANTIBACTERIAL ACTIVITY

Therapeutic Activity. The therapeutic efficacy of colistin^{22, 64, 68} and the polymyxins^{60, 61, 63, 69, 70} in several experimental gram-negative bacterial infections in mice has been amply demonstrated. The relative effectiveness of colistin sulfate, sodium colistinmethanesulfonate, and polymyxin B sulfate was assessed in a side-by-side comparison against two experimental infections in mice. The median curative doses (CD_{50}) were determined with acute infections in mice by intraperitoneal injection of *K. pneumoniae* 1 and *E. coli* "J" followed by subcutaneous or oral treatment with graded doses of aqueous solutions of the antibiotics. A typical experiment and protocol are shown in table V.

Quantitative evaluation of the data from a number of experiments, based on dose-effect curve parameters⁷¹ showed that there were significant differences between these three antibiotics. The results of these quantitative comparisons against *K. pneumoniae* are shown in table VI and those for *E. coli*, in table VII. Sodium colistinmethanesulfonate was the most active of the three against *K. pneumoniae* by subcutaneous route. Colistin sulfate and polymyxin B sulfate showed approximately equal activity by the subcutaneous route. Orally, colistin sulfate was found to be the most active, with no significant differences in activity between the other two.

With respect to the *E. coli* infection, colistin sulfate was the most effective on a dosage basis, by both the subcutaneous and oral routes. Polymyxin B sulfate and

sodium colistinmethanesulfonate were equally active subcutaneously; however, sodium colistinmethanesulfonate was more active by the oral route.

RESISTANCE STUDIES

The emergence of antibiotic-resistant strains, plus the occurrence of cross resistance among antibiotics, has greatly narrowed the usefulness of such agents. The problem of acquired drug resistance is not peculiar to antibiotic therapy, for this phenomenon has been encountered in varying degrees with all chemotherapeutic agents. Several investigators^{1, 38, 41} have reported that sensitive organisms do not readily develop resistance to colistin and that there is no cross resistance with the broad-spectrum antibiotics. Moreover, the majority of induced resistant mutants are not stable and readily revert in the absence of colistin. The low incidence of development of resistance and the same difficulties in developing and maintaining resistant strains to polymyxins have also been reported.^{62, 63, 70, 72} Current in vitro and in vivo studies with colistin sulfate have shown essentially the same results. Since a detailed report of these studies with colistin and other antibiotics is in preparation, only pertinent results and conclusions are presented at this time. These, of course, are valid only for the sensitive, induced resistant, and naturally resistant strains that were studied.

In Vitro Studies. STAPHYLOCOCCUS AUREUS STRAINS. These strains developed a

TABLE VII
Relative Activity of Colistin Sulfate, Sodium Colistinmethanesulfonate, and Polymyxin B Sulfate in E. coli "J" Infections in Mice (Summary)

Antibiotic	Median effective dose,* mg./Kg.	Slope function†	Relative activity‡
<i>Four Subcutaneous Dosage Schedule</i>			
Polymyxin B sulfate	9.8 (7.7–12.45)	1.61 (1.30–1.98)	1.00
	8.0 (5.7–11.2)	1.99 (1.20–3.28)	1.22
Colistin sulfate	2.78 (1.24–4.07)	2.10 (1.00–4.41)	3.52
	2.5 (1.67–3.75)	2.33 (1.55–3.50)	3.92
Sodium colistinmethanesulfonate	8.2 (6.45–10.41)	1.77 (1.13–2.74)	1.19
	7.5 (5.64–9.98)	1.77 (1.52–2.05)	1.30
<i>Single Subcutaneous Dosage Schedule</i>			
Polymyxin B sulfate	12.4 (8.2–18.72)	2.0 (0.2–20.0)	1.00
Colistin sulfate	3.0 (1.87–4.8)	1.90 (0.19–19.0)	4.13
Sodium colistinmethanesulfonate	10.7 (8.04–14.23)	2.10 (1.26–3.46)	1.15
<i>Four Oral Dosage Schedule</i>			
Polymyxin B sulfate	590. (347.–1,003.)	2.35 (0.9–6.11)	1.00
Colistin sulfate	138.5 (86.5–221.6)	1.95 (1.24–3.09)	4.25
Sodium colistinmethanesulfonate	167.0 (115.–242.2)	2.10 (1.58–2.91)	3.53

* Figures in parentheses are 95 per cent confidence limits.

† Slope function = $\frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$.

‡ Relative activity = ED_{50} of polymyxin B sulfate/ ED_{50} of the test antibiotic.

moderate resistance to colistin sulfate and a high resistance to penicillin. In one strain the development of resistance to penicillin resulted in a collateral sensitivity to colistin sulfate; in another strain the resistance to colistin sulfate resulted in a limited cross resistance to penicillin. The inhibition or repression of the emergence of resistance to penicillin was demonstrated in some instances with fixed subinhibitory concentrations of colistin sulfate.

E. COLI STRAINS. These strains ordinarily developed a rapid and high resistance to streptomycin, but no significant resistance to colistin sulfate or polymyxin B sulfate. The strains that developed resistance to streptomycin usually showed no cross resistance to colistin sulfate, polymyxin B sulfate, or penicillin. Collateral sensitivity to colistin sulfate, polymyxin B sulfate, and penicillin was occasionally observed.

The influence of colistin sulfate on the emergence of resistance to streptomycin was variable and different with the various species. The inhibition or depression of resistance could only be demonstrated with particular concentration ratios of the two antibiotics.

In Vivo Studies. An increase in resistance of *E. coli* "J" and *K. pneumoniae* 1 to streptomycin sulfate and colistin sulfate was demonstrated after serial passage in mice being treated subcutaneously with less than effective dosages of these antibiotics (evidence from comparative CD_{50} values of original and passage strains). With oral administration of these antibiotics, *K. pneumoniae* 1 developed resistance to both, whereas *E. coli* "J" developed resistance only to streptomycin. Subcutaneous administration of combinations of these antibiotics inhibited the emergence of resistant strains of *E. coli* "J." The strains that appeared resistant to colistin sulfate when tested in vivo were sensitive when tested in vitro. Most of the strains made resistant to streptomycin in vivo proved to be resistant also when tested in vitro.

BLOOD LEVELS

The method used to determine the concentrations of colistin in serum and other body fluids was essentially the cylinder plate assay for polymyxin using *Bordetella bronchiseptica* ATCC 4617 as the test organism.⁷³ In general, serial dilution techniques with sensitive gram-negative organisms are less sensitive and more subject to variation due to the presence of antibodies for gram-negative organisms.

Parenteral Administration. Studies were conducted with rabbits, dogs, and human beings using single intramuscular doses of sodium colistinmethanesulfonate representing 16.6 to 166.5 mg. base per animal and with rabbits using 33.3 to 166.5 mg. base of colistin sulfate per animal. The data are presented in table VIII.

Significant blood serum levels were obtained rapidly with sodium colistinmethanesulfonate at all dosage levels in all three species. In rabbits, doses equal to 12, 24, and 60 mg. base per Kg. maintained blood serum concentrations greater than 0.5 $\mu\text{g./ml.}$ for at least 48 hours. A dose equal to 0.56 mg. base per Kg. gave low but significant serum levels in the dog for six hours, while 1.15 mg. base per Kg. produced high levels for more than six hours but no detectable amounts at 24 hours. Single total doses equal to 16.6 and 33.3 mg. base provided detectable blood serum levels up to eight hours in human beings. Single therapeutic doses equal to 83.2 and 166.5 mg. base gave high initial blood serum levels in man, which were maintained at concentrations greater than 10 $\mu\text{g./ml.}$ for six hours. Since these patients were

TABLE VIII

Blood Serum Concentrations after Single Intramuscular Doses of Sodium Colistinmethanesulfonate and Colistin Sulfate in Rabbits, Dogs, and Human Beings

Species	No. animals	Total dose, mg. base	Average dose, mg. Kg.	Average serum levels, $\mu\text{g.}/\text{ml.}$, at following hour*									
				1/2-1	2	4	6	8	10	12	24	36	48
<i>Sodium Colistinmethanesulfonate</i>													
Rabbit	2	166.5	60.0	—	1.02	0.97	0.86	0.85	0.85	0.84	0.76	0.72	0.69
	4	83.2	24.0	—	2.05	1.97	1.81	1.70	1.54	—	1.43	1.15	0.99
	8	33.3	12.0	—	2.22	1.91	1.85	1.72	1.56	—	1.38	1.28	0.87
Dog	1	33.3	1.15	6.6	6.6	6.6	2.5						
	1	16.6	0.56	0.26	0.26	0.26	0.13						
Human being	1	166.5		—	>53.3	>53.3	>53.3						
	1	83.2		—	>10.6	>10.6	>10.6						
	4	33.3		—	1.36	1.08	0.64	0.30					
	4	16.6		—	0.47	0.03	0.26	0.14					
<i>Colistin Sulfate</i>													
Rabbit	4	166.5	60.0	—	11.98	6.35	3.07	1.26	0.90	0.25	0	0	0
	4	83.2	30.0	—	2.05	1.88	1.78	1.76	1.56	—	1.52	1.41	1.26
	6	33.3	11.0	—	1.55	1.46	1.38	1.27	1.21	—	1.05	0.93	0.72

*All of the pretreatment sera were negative.

on a multiple dose schedule for tolerance studies, subsequent hourly determinations were not included in these results.

Single intramuscular doses of colistin sulfate representing 11 and 30 mg. base per Kg. in rabbits gave blood serum concentrations greater than 0.5 $\mu\text{g.}/\text{ml.}$ for 48 hours; 60 mg. base per Kg. equivalent gave exceedingly high levels up to four hours, but no detectable amounts were evident at 24 hours after injection. The high blood serum levels and rapid disappearance with this dose may be related to some of the pharmacological actions of this particular form of the antibiotic.

Blood serum and subretinal fluid levels were determined in 18 patients after the intramuscular administration of single doses of sodium colistinmethanesulfonate equal to 33.3 mg. base. The results are shown in table IX. Detectable blood serum levels of the antibiotic were demonstrated in all patients but only 7 of them showed subretinal fluid levels 3 to 4.5 hours after treatment.

Oral Administration. Oral studies in rabbits with sodium colistinmethanesulfonate and colistin sulfate showed poor and variable absorption after the administration of doses equal to 60 mg. base per Kg.

PHARMACOLOGY

Acute Toxicity. Studies were undertaken to compare the acute toxicities of colistin sulfate, sodium colistinmethanesulfonate, and polymyxin B sulfate after various routes of administration in mice. At least 10 animals of similar weights were employed at each dosage level. The LD_{50} values for the two forms of colistin are presented in table X.

The results of the acute toxicity determinations in mice demonstrated that sodium colistinmethanesulfonate was the least toxic of the three antibiotics by three routes of administration. Similar values were obtained for colistin sulfate and polymyxin B

TABLE IX

Serum and Subretinal Fluid Concentrations after Single Intramuscular Dose (33.3 mg. Base) of Sodium Colistinmethanesulfonate in Human Beings

Patient*	Hours after administration	Level, $\mu\text{g./ml.}$	
		Serum	Subretinal fluids
1	2.5	0.20	0
2	3.0	0.63	0-0.16
3	3.0	0.40	0
4	3.25	—	0.20
5	3.75	—	0.20
6	3.75	—	0.26
7	3.75	0.26	0
8	4.0	0.56	0
9	4.0	0.80	0
10	4.25	0.80	0
11	4.25	0.80	0-0.16
12	4.50	0.73	0-0.08
13	4.50	1.46	0
14	4.75	0.28	0-0.16
15	4.75	0.50	0
16	5.0	0.46	0
17	5.25	0.36	0
18	5.25	0.28	0
Controls	(5)	0	0

* Studies carried out in cooperation with Dr. Lemuel T. Moorman, Massachusetts Eye and Ear Infirmary, Boston.

sulfate in most instances. The low toxicity of sodium colistinmethanesulfonate after intravenous administration appeared to be correlated with its rapid elimination by the kidneys.

Brownlee et al⁶⁰ reported that all polymyxins showed similar acute toxicities in mice and rabbits using several samples of varying potency and purity.

Numerous other reports^{61, 63, 69, 72, 74-76} on the acute toxicity of the polymyxins in several species after various routes of administration have shown variation in the LD₅₀ values.

Cardiovascular Effects. A comparative study was made to determine the effects of colistin sulfate and sodium colistinmethanesulfonate on the circulation of dogs given pentobarbital. It has been reported^{60, 74} that polymyxin B causes inconsistent responses, which vary from no effect to sharp falls in the blood pressure of this species.

TABLE X

Comparative LD₅₀ Values

Route	Colistin sulfate, mg. base/Kg.	Sodium colistin-methanesulfonate, mg. base/Kg.	Polymyxin B sulfate, mg. polymyxin standard/Kg.
Intravenous	5.46	222.33	5.40
Intraperitoneal	19.80	126.50	20.50
Subcutaneous	48.60	138.00	59.50
Oral (4 hr. fasted)	720.00	>766.67	790.00

TABLE XI

Cardiovascular Responses to Colistin

Antibiotic	Dose, mg./Kg., intravenous	Blood pressure response, mm. Hg	Per cent change	Duration of response, minutes
Colistin sulfate	0.5	10.0—	7.0—	2.0
	1.0	17.5—	11.0—	10.0
	2.0	80.0—	53.0—	>60.0
	6.5	110.0—	83.0—	>60.0
Sodium colistin- methanesulfonate	4.5	0	0	—
	6.5	5.0—	3.0—	<2.0

The doses of colistin sulfate, expressed as the salt, were administered intravenously over a two minute period, while those of sodium colistinmethanesulfonate, also expressed as the salt, were given within a 10 second period. Table XI summarizes the results obtained with various dosage levels.

Despite the fact that the 6.5 mg. dose of sodium colistinmethanesulfonate has approximately two times the base potency of the 2 mg. dose of colistin sulfate, there was a pronounced difference in the effect on the blood pressure of the anesthetized dog. No information is available to explain the mechanism of the increasing depressor responses elicited by graded doses of colistin sulfate.

Isolated Tissue Effects. Isolated tissue studies, using rabbit and guinea pig ileum, showed that sodium colistinmethanesulfonate produced an inconsistent response, which varied from a slight spasmogenic effect at concentrations of 5 µg./ml. of bath solution to no effect in concentrations as high as 75 µg./ml. Colistin sulfate caused no effect on normal gut motility in concentrations up to 50 µg./ml. of bath solution. Neither antibiotic had any effect on the spasms induced by acetylcholine, barium chloride, or histamine.

Irritation Studies. The daily intraperitoneal administration of 1.3 and 3.9 mg. base per Kg. of colistin sulfate for seven days in mice gave no evidence of irritation; 33.3 mg. base of sodium colistinmethanesulfonate administered intramuscularly twice daily to dogs for two weeks caused no gross or microscopic evidence of tissue damage. Single doses of sodium colistinmethanesulfonate (166.6 mg. base) were well tolerated by dogs, and none of the animals exhibited any pain response.

Subacute Studies. Sodium colistinmethanesulfonate* was administered intraperitoneally to rats and intramuscularly to dogs in doses equal to 0.83, 2.5, and 7.5 mg. base per Kg. daily for 60 days. The rats showed no significant adverse effects in growth, behavior, hematological findings, or urinalyses. A similar finding was noted in the dog experiments with the exception of some tenderness at the injection sites in 2 of 4 animals at the middle dosage level and 3 of 4 at the high dosage level.

Examination of the tissue microsections from the rats revealed a subacute inflammatory process in the small intestine occurring at all dosage levels but that was associated with definite superficial erosion only at the highest dosage level. Focal, marked, acute, passive hyperemia of kidneys was present regularly in high dosage animals and infrequently in lower dosage animals. There appeared to be a dose re-

* A commercial preparation containing dibucaine and phosphate buffer prepared by Kayaku Antibiotic Research Company, Ltd., Tokyo, Japan, was used in this part of the study.

lationship with respect to the renal changes; however, they were considered to be of minimal significance. The tissues from the dogs revealed no changes attributable to the sodium colistinmethanesulfonate.

Colistin sulfate was administered orally to rats and dogs in doses equal to 6.67, 20, and 60 mg. base per Kg. daily for 90 days. None of the three dosage levels caused any adverse effect on growth, behavior, urinalyses, liver or kidney function (dogs), or hematological findings in either species.

Microscopic examination of the tissues from these animals did not reveal any pathological changes that could be attributed to drug administration.

SUMMARY AND CONCLUSIONS

1. Colistin sulfate and sodium colistinmethanesulfonate, two forms of a new polypeptide antibiotic, colistin, showed marked antibacterial activity in vitro against a wide spectrum of gram-negative organisms and lesser activity against gram-positive organisms and fungi.

2. Both substances demonstrated bactericidal, as well as bacteriostatic, activity in vitro in similar concentrations against susceptible organisms. This activity was not significantly reduced in the presence of serum nor by changes in pH between 6.0 and 8.0. In vitro activity was not appreciably decreased by increasing the size of the inoculum from 10^3 to 10^7 organisms.

3. Similar in vitro activities were demonstrated for colistin sulfate and polymyxin B sulfate, while sodium colistinmethanesulfonate was slightly less active in most instances. With the possible exception of *Proteus* strains, these substances were generally more active than streptomycin against pathogenic gram-negative bacilli.

4. Resistance to colistin sulfate was only occasionally induced with difficulty in sensitive bacterial strains. Most of the induced resistant forms were not stable and reverted in the absence of the antibiotic. Strains made resistant to streptomycin or other broad-spectrum antibiotics were not cross resistant to colistin.

5. A side-by-side comparison of the therapeutic efficacy against two experimental infections in mice showed that colistin sulfate was more effective orally against *E. coli* and *K. pneumoniae* than sodium colistinmethanesulfonate and polymyxin B sulfate. By the subcutaneous route, sodium colistinmethanesulfonate was the most effective agent against *K. pneumoniae* while colistin sulfate was most effective against *E. coli*.

6. Adequate single doses of sodium colistinmethanesulfonate administered intramuscularly to rabbits, dogs, and human beings produced rapid and significant blood serum levels, which varied in duration with the administered dose. Detectable levels were noted in the subretinal fluid at 3 to 4.5 hours in 7 of 18 patients given an intramuscular dose of 33.3 mg. base.

7. Oral studies with colistin sulfate and sodium colistinmethanesulfonate in rabbits showed poor and variable absorption.

8. The results of acute toxicity determinations in mice demonstrated that sodium colistinmethanesulfonate was less toxic than colistin sulfate and polymyxin B sulfate by the intravenous, intraperitoneal, and subcutaneous routes. Similar values were obtained for colistin sulfate and polymyxin B sulfate in most instances.

9. Sodium colistinmethanesulfonate did not cause significant vasodepression in

large intravenous doses, while colistin sulfate gave increasing depressor effects in doses of 1 to 6.5 mg./Kg.

10. Sodium colistinmethanesulfonate proved to be occasionally spasmogenic in isolated tissue studies, while colistin sulfate was without effect in concentrations up to 50 µg./ml. of bath solution.

11. Sodium colistinmethanesulfonate (33.3 mg. base) administered intramuscularly twice daily for 14 days was well tolerated in dogs and showed no microscopic evidence of damage. High single doses (166.6 mg. base) were equally well tolerated in this species.

12. Sodium colistinmethanesulfonate administered intraperitoneally to rats and intramuscularly to dogs in doses equal to 0.83, 2.5, and 7.5 mg. base/Kg. daily for 60 days caused no adverse effects on growth, behavior, liver, and kidney function (dogs), urinalyses, or hematological findings. Some subacute inflammation was noted in the small intestine of rats at all dosage levels, and some focal acute passive hyperemia was present regularly in the kidneys of this species at the high dosage level but infrequently in the lower dosage animals. No tissue changes that could be attributed to the drug were evident in the dogs.

13. The daily oral administration of colistin sulfate to rats and dogs for 90 days failed to show any adverse effect in doses equal to 6.67, 20, and 60 mg. base per Kg.

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Chemical, Biological, and Clinical Observations on Colistin

WILLIAM W. WRIGHT AND HENRY WELCH

*U. S. Department of Health, Education, and Welfare,
Food and Drug Administration, Washington, D. C.*

Colistin,* an antibiotic produced by *Aerobacillus colistinus*, was first reported in 1950 by Koyama et al.¹ It is highly inhibitory for many gram-negative organisms²⁻⁶ and only slightly inhibitory or inactive toward gram-positive organisms. Colistin resembles the polymyxins⁷ and circulin⁸ in its chemical composition in that it contains amino acids, α , γ -diaminobutyric acid and methyl octanoic acid.

This paper describes some observations on the composition and reactions of authentic samples of colistin, the polymyxins, and circulin. Because of their similar composition and spectrum of antibacterial activity, colistin sulfate and polymyxin B sulfate were compared for their in vitro inhibitory action against 58 strains of bacteria. Pharmacological studies were made on the acute toxicity in mice and rats of colistin sulfate and a derivative, colistinmethanesulfonate sodium. In addition, clinical studies were conducted in man to determine blood concentrations and urinary excretion after various doses of colistinmethanesulfonate sodium administered orally and by intramuscular injection.

EXPERIMENTAL

The following materials were studied: Colistin sulfate lot CB-7710 (Kayaku) with a potency of 18,000 units/mg. was tested. Pure colistin base contains 30,000 units/mg. The theoretical potency for pure colistin sulfate is believed to be 24,000 units/mg. (800 μ g. of colistin base per mg.). Throughout this paper any weight of a colistin preparation referred to is the amount of colistin base in such preparation. Lot CB-7710 thus has a potency of 600 μ g./mg. and is about 75 per cent pure. This was used as the standard of comparison in all colistin assays. The colistin sulfate master standard (20,000 units/mg.) was tested only for amino acid content. Colistinmethanesulfonate sodium was studied as a pure compound and as a formulation for injection. The pure compound was lot 2167 and assayed 444 μ g. of colistin per mg. The formulation for injection contained for each 30.0 mg. of colistin activity, 4 mg. of monobasic potassium phosphate, 2 mg. of dibasic sodium phosphate, and 2 mg. of dibucaine hydrochloride. Three lots of this mixture were employed (GMC-1001, GMC-1002, and GMC-5001). By actual assay, lot GMC-1002 contained 407 μ g./mg.

Polymyxin A sulfate lot P-14-1 (Pfizer), when assayed by the microbiological plate assay,⁹ had an activity equivalent to 12,000 units of polymyxin B per mg. Polymyxin B sulfate used in the chemical studies was the U.S.P. reference standard with a potency of 7850 units/mg. Lot P42M with a potency of 7500 units/mg. (750 μ g./mg.) was used in the in vitro susceptibility tests. Lot P4P with a potency of 7200 units/mg. (720 μ g./mg.) was used in the acute toxicity tests. Polymyxin E

* The trade name of Warner-Chilcott Laboratories for colistin sulfate is Coly-mycin S; for colistinmethanesulfonate sodium, Coly-mycin M.

sulfate lot q-257 N.D. 117, polymyxin E base (Wilkinson, 12,070 units/mg.), and polymyxin E base lot A458/10/2 (9804 units/mg.) were tested only for L-leucine and L-threonine content and there was insufficient material to do other tests. Circulin res. no. 8966 (antibiotic Q19, Upjohn) with an activity equivalent to 8250 units of polymyxin B per mg.⁹ was studied.

Chemical Studies. Infrared absorption spectra were obtained using potassium bromide discs containing 0.5 per cent of colistin sulfate or polymyxin B sulfate. Aqueous solutions (5 mg./ml.) were tested for ultraviolet absorption. Colistin sulfate, polymyxin A, and circulin were tested by descending paper chromatography for 24 to 48 hours at 25 C. using as solvents butanol saturated with 2 per cent aqueous acetic acid or butanol saturated with an aqueous solution containing 20 per cent acetic acid and 10 per cent pyridine. Acid hydrolysates were prepared by heating 2 to 5 mg. in 1 ml. of 6 N hydrochloric acid for four to seven hours at 120 C. in an autoclave. The hydrolysates were tested for the presence of leucine, phenylalanine, serine, and threonine, using ascending paper chromatography with 80 per cent phenol or butanol saturated with 25 per cent aqueous acetic acid as solvents. All paper chromatograms were sprayed with a solution of 1 per cent ninhydrin in butanol saturated with water and developed by heating at 100 C. for five minutes or allowing to stand overnight at room temperature. Acid hydrolysates of colistin sulfate, polymyxins A and E, and circulin were assayed quantitatively by microbiological assays¹⁰ for L-leucine and L-threonine.

In Vitro Susceptibility Tests. Fifty-eight strains of bacteria were tested for susceptibility to colistin sulfate and polymyxin B sulfate by the tube serial dilution technique. Trypticase soy broth was used. The minimal inhibitory concentration after 18 hours' incubation at 37 C. was recorded as the bacteriostatic concentration. All tubes showing inhibition at this time were subcultured by making a 1:15 dilution in Trypticase soy broth and incubating 18 hours at 37 C. The concentration from which no viable organisms were recovered was recorded as the bactericidal concentration.

Toxicity. White Swiss mice of both sexes and young adult Osborne-Mendel rats (about 200 Gm.) were used. The acute toxicity of colistin sulfate and colistinmethanesulfonate sodium was determined using one or more of the following routes of administration: intraperitoneal, subcutaneous, intravenous, intramuscular, oral. In some cases direct comparisons were made with polymyxin B sulfate. Short-term chronic toxicity was determined intraperitoneally, intramuscularly, and orally by administering two doses daily (morning and afternoon) until seven doses had been given. The drugs were dissolved in physiological saline solution in usual concentrations of 0.2 per cent for mice and 1 per cent for rats. Examinations were made of representative microscopic sections of kidney, liver, spleen, stomach, and skeletal muscle.

The swimming test¹¹ for neurotoxicity in mice was used to compare colistin sulfate and polymyxin B sulfate. Fifteen mice weighing from 19 to 21 Gm. were used for each antibiotic. A control group of 10 mice received physiological saline solution.

CLINICAL STUDIES

Intramuscular Injection. Ten healthy adult men received a single injection of 15 mg. of colistin (as the methanesulfonate) in a volume of 2 ml. Ten men received

30 mg. in 2 ml. Five received 75 mg. in 2.5 ml. and 10 received 150 mg. in 5 ml. Sterile distilled water for injection was used as the diluent in every case. In a study of multiple doses, 5 men received 15 mg. followed six hours later by another 15 mg.; another 5 men received 30 mg. doses spaced six hours apart.

Oral Administration. Five men received a single oral dose of two hand-filled capsules each containing 272 mg. of the injectable formulation—a total of 221.4 mg. of colistin (as colistinmethanesulfonate). Five men received a single 750 mg. dose of colistinmethanesulfonate (six 125 mg. capsules) and 5 men received a single 1500 mg. dose.

Blood Concentrations. Determinations of blood serum concentration were made immediately before each single dose injection and at two, four, six, and eight hours after the injection. After the 75 and 150 mg. doses, serum concentrations were also determined at 12 and 24 hours, except for one group of 5 subjects receiving the 150 mg. dose, in which case concentrations were determined only at 12, 14, 16, 18, 20, 22, and 24 hours. In the multiple dose study, serum concentrations were determined just before and four hours after the first injection, and at two and six hours after the second injection. Blood specimens were obtained immediately before and at two, four, six, and eight hours after the oral dose. The sera were assayed by a microbiological method similar to the one described for polymyxin B using *Bordetella bronchiseptica* ATCC 4617 as the test organism.⁹ A single 10 ml. seeded base agar layer was used and the serum specimens were diluted with an equal volume of 20 per cent phosphate buffer pH 6 (4 Gm. dipotassium phosphate, 16 Gm. monopotassium phosphate, and sufficient distilled water to make 100 ml.). The standard response line was constructed using colistin sulfate dissolved in a diluent composed of half normal human serum and half 20 per cent phosphate buffer pH 6. The concentrations used were 0.1, 0.2, 0.4, 0.8, and 1.6 $\mu\text{g./ml.}$, with the reference point being 0.4 $\mu\text{g./ml.}$

Urinary Excretion. Control urine specimens were obtained just before the single injections of 15 and 30 mg. and before the oral dose of 221 mg. All the urine was then collected and the volumes measured in the time intervals of zero to two, two to four, four to six, and six to eight hours after the medication.

Urines were assayed by a microbiological method similar to the one described for polymyxin,⁹ except that the standard response line was prepared in 10 per cent phosphate buffer pH 6 to obtain concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu\text{g.}$ of colistin per ml. The reference concentration was 1.0 $\mu\text{g./ml.}$ The urines were prepared for assay by diluting with an equal volume of 20 per cent phosphate buffer pH 6. If concentrations in excess of 8 $\mu\text{g./ml.}$ were anticipated, the samples were further diluted with 10 per cent phosphate buffer pH 6 to obtain an estimated final concentration of 1.0 $\mu\text{g./ml.}$

RESULTS

Chemical Studies. The infrared absorption spectrum of colistin sulfate was almost identical with that given by polymyxin B sulfate. Both antibiotics had absorption maxima at 3.08, 3.28, 3.40, 6.00 (strong), 6.50 (strong), 6.80, 7.20, 8.05, 9.00 (broad), and 10.28 μ . Polymyxin B sulfate absorbed weakly at 14.28 μ , but colistin sulfate did not. In aqueous solution neither compound exhibited any

TABLE I
Amino Acids in Colistin, Circulin, and the Polymyxins

Amino acid	Colistin sulfate			Circulin		Polymyxin A		Polymyxin B		Poly- myxin C	Poly- myxin D	Polymyxin E			
	Refer- ence ¹	Found [†]		Refer- ence ¹	Found	Refer- ence ¹	Found	Refer- ence ¹	Found	Refer- ence ¹	Refer- ence ¹	Refer- ence ¹	Found [‡]		
		1	2										1	2	3
D-Leucine	11.8*	NT [†]	NT	+	NT	+	NT	NS	NT	0	+	+	NT	NT	NT
L-Leucine	11.8	8.0	7.92	NS	1.35	NS	0.61	+	+	NS	0	NS	6.40	9.6	10.2
L-Phenyl- alanine	0	0	NT	0	NT	0	NT	+	+	+	0	0	NT	NT	NT
L-Serine	0	0	NT	0	NT	0	NT	0	0	0	+	0	NT	NT	NT
L-Threonine	9.9	12.6	15.1	+	7.6	+	11.2	+	+	+	+	+	20.9	18.0	18.0

* Per cent by weight.

[†] Colistin sample 1 is lot CB-7710, sample 2 is colistin sulfate master standard.

[‡] NT = Not tested. NS = Not stated. + = Present. 0 = Not present.

[§] Polymyxin E sample 1 is lot q-257 N.D. 117, sample 2 is the Wilkinson lot, and sample 3 lot A458/10/2.

characteristic ultraviolet absorption peaks, with only end absorption in the 210 to 250 m μ region being observed.

Using descending paper chromatography, colistin sulfate revealed two fast-moving fractions; circulin, two fractions moving almost as fast as those of colistin; and polymyxin A sulfate, a single slow-moving component. A mixture of colistin and circulin was resolved incompletely, since only three fractions were observed, one believed to be from colistin, one from circulin, and one possibly a common component. A mixture of colistin sulfate and polymyxin A sulfate was completely resolved.

Ascending paper chromatography of the acid hydrolysates revealed the presence of leucine and threonine in colistin sulfate and confirmed the absence of serine and phenylalanine. Polymyxin B sulfate contained leucine, threonine, and phenylalanine, but no serine.

The amino acid contents found for colistin, circulin, and the polymyxins are given in table I, together with data reported in the literature.

In Vitro Susceptibility Studies. The bacteriostatic and bactericidal concentrations of colistin sulfate and polymyxin B sulfate are given in table II. Both antibiotics were highly inhibitory for the coli-aerogenes, *Pseudomonas*, *Salmonella*, and *Shigella* groups of organisms. In every case colistin was more active than polymyxin B. The bactericidal concentrations were usually approximately twice the bacteriostatic amounts. Both colistin and polymyxin B were practically inactive against strains of *Proteus*, staphylococci, and streptococci.

Toxicity. COLISTIN SULFATE. Intraperitoneally the acute LD₅₀ of colistin sulfate was 20.8 mg. of the base per Kg. for mice and 9.6 mg./Kg. for rats. The intravenous LD₅₀ was 5.43 mg./Kg. in mice, and subcutaneously it was 51.6 mg./Kg. Administered orally to rats, the LD₅₀ of colistin sulfate was 110.4 mg./Kg. The comparative LD₅₀ values for polymyxin B sulfate in mice were 21 mg./Kg. (intraperitoneal), 5.87 mg./Kg. (intravenous), and 64.7 mg./Kg. (subcutaneous).

In a short-term chronic toxicity test, 5 mice survived 9.6 mg./Kg. intraperitoneally twice daily for seven doses, and 10 mice survived 9.6 mg./Kg. intramuscularly twice daily for 10 days. Five rats received 4.8 mg./Kg. intraperitoneally

TABLE II

Susceptibility of 58 Bacterial Cultures to Colistin and Polymyxin

Organism		Bacteriostatic, $\mu\text{g.}/\text{ml.}$		Bactericidal, $\mu\text{g.}/\text{ml.}$	
		Colistin	Polymyxin	Colistin	Polymyxin
<i>Aerobacter aerogenes</i>	ATCC 8308	0.6*	1.5†	1.2	6.0
<i>Aerobacter aerogenes</i>	ATCC 8329	1.2	3.0	1.2	12.0
<i>Aerobacter aerogenes</i>	ATCC 8724	1.2	3.0	2.4	6.0
<i>Aerobacter aerogenes</i>	ATCC 9621	1.2	3.0	1.2	6.0
<i>Aerobacter aerogenes</i>	ATCC 884	0.3	3.0	0.6	3.0
<i>Escherichia coli</i>	ATCC 26	0.6	3.0	0.6	12.0
<i>Escherichia coli</i>	ATCC 206	0.6	1.5	2.4	3.0
<i>Escherichia coli</i>	ATCC 133	0.6	1.5	0.6	6.0
<i>Escherichia coli</i>	ATCC 4692	0.6	1.5	2.4	6.0
<i>Escherichia coli</i>	ATCC 10053	0.3	1.5	0.3	12.0
<i>Pseudomonas aeruginosa</i>	DA 813	≤ 0.15 ‡	3.0	0.6	6.0
<i>Pseudomonas aeruginosa</i>	DA 815	0.3	1.5	1.2	3.0
<i>Pseudomonas aeruginosa</i>	DA 816	≤ 0.15	1.5	> 0.6 §	3.0
<i>Pseudomonas atrofaciens</i>	ATCC 9004	≤ 0.15	1.5	> 0.6	3.0
<i>Pseudomonas species</i>	DA 837	≤ 0.15	1.5	> 0.6	3.0
<i>Salmonella typhosa</i>	DA 420B	≤ 0.15	0.75	≤ 0.15	1.5
<i>Salmonella typhosa</i>	DA 426	≤ 0.15	0.75	0.3	1.5
<i>Salmonella typhosa</i>	DA 428	0.3	0.38	0.3	0.75
<i>Salmonella typhosa</i>	ATCC 8304	≤ 0.15	0.75	0.6	1.5
<i>Salmonella typhosa</i>	ATCC 6539	≤ 0.15	0.38	≤ 0.15	0.75
<i>Salmonella urbana</i>	DA 917	≤ 0.15	3.0	0.6	12.0
<i>Salmonella typhimurium</i>	DA 918	≤ 0.15	0.75	0.3	3.0
<i>Salmonella paratyphi A</i>	DA 919	≤ 0.15	0.75	≤ 0.15	1.5
<i>Salmonella schottmülleri</i>	DA 922	≤ 0.15	1.5	0.6	3.0
<i>Shigella flexneri</i>	DA 953	≤ 0.15	0.75	0.15	0.75
<i>Shigella flexneri</i>	DA 969	≤ 0.15	0.38	0.3	0.38
<i>Shigella sonnei</i>	DA 959	≤ 0.15	0.38	≤ 0.15	0.75
<i>Shigella paradysenteriae</i>	ATCC 9380	≤ 0.15	≤ 0.19	0.6	0.75
<i>Proteus vulgaris</i>	ATCC 881	> 300	> 376	—	—
<i>Proteus vulgaris</i>	ATCC 6380	> 300	> 376	—	—
<i>Proteus vulgaris</i>	ATCC 6896	> 300	> 376	—	—
<i>Proteus vulgaris</i>	ATCC 7289	> 300	> 376	—	—
<i>Proteus vulgaris</i>	ATCC 9623	> 300	> 376	—	—
<i>Proteus mirabilis</i>	DA 759	> 300	> 376	—	—
<i>Proteus mirabilis</i>	DA 760	> 300	> 376	—	—
<i>Proteus mirabilis</i>	DA 764	> 300	> 376	—	—
<i>Proteus mirabilis</i>	DA 765	> 300	> 376	—	—
<i>Staphylococcus aureus</i>	ATCC 6538P	150	94	> 300	376
<i>Staphylococcus aureus</i>	DA 303	150	94	—	—
<i>Staphylococcus aureus</i>	DA 304	150	94	—	—
<i>Staphylococcus aureus</i>	DA 306	150	94	—	—
<i>Staphylococcus aureus</i>	DA 307	300	94	—	—
<i>Staphylococcus aureus</i>	DA 308	300	94	—	—
<i>Staphylococcus aureus</i>	DA 310	300	94	—	—
<i>Staphylococcus aureus</i>	DA 311	300	188	—	—
<i>Staphylococcus aureus</i>	DA 305	75	94	—	—
<i>Staphylococcus aureus</i>	DA 309	150	47	—	—
<i>Staphylococcus aureus</i>	DA 315	300	188	—	—

Table II Continued on Page 66

TABLE II (Continued)
Susceptibility of 58 Bacterial Cultures to Colistin and Polymyxin

Organism		Bacteriostatic, $\mu\text{g.}/\text{ml.}$		Bactericidal, $\mu\text{g.}/\text{ml.}$	
		Colistin	Polymyxin	Colistin	Polymyxin
<i>Staphylococcus faecalis</i>	ATCC 6569	>300	>376	—	—
<i>Staphylococcus faecalis</i>	ATCC 7080	>300	>376	—	—
<i>Staphylococcus faecalis</i>	ATCC 8022	>300	>376	—	—
<i>Staphylococcus faecalis</i>	ATCC 9790	>300	>376	—	—
<i>Staphylococcus faecalis</i>	ATCC 10541	>300	>376	—	—
<i>Streptococcus viridans</i>	ATCC 9756	>300	>376	—	—
<i>Streptococcus viridans</i>	ATCC 9758	>300	>376	—	—
<i>Streptococcus viridans</i>	DA 1300	>300	>376	—	—
<i>Streptococcus viridans</i>	ATCC 7073	150	94	—	—
<i>Streptococcus viridans</i>	ATCC 9222	37.5	47	—	—

* $\mu\text{g.}$ of colistin base per ml.

† $\mu\text{g.}/\text{ml.}$ of base. Since Lot P42-M had a potency of 7500 units/mg. and the theoretical value for the base is 10,000 units/mg., the lot contained 750 $\mu\text{g.}/\text{mg.}$

‡ Since 0.15 $\mu\text{g.}/\text{ml.}$ was the lowest concentration employed, when a culture was inhibited by it the result was recorded as equal to or less than 0.15 $\mu\text{g.}/\text{ml.}$

§ The test for bactericidal activity was done by transferring the contents of each of the first three tubes in the series showing inhibition (bacteriostatic test) to a tube containing 15 ml. thioglycollate broth. Hence, when all three tubes showed growth in the bactericidal test the result was recorded as greater than the highest concentration tested.

|| Except for ATCC 6538P, cultures inhibited by 37.5 $\mu\text{g.}/\text{ml.}$ or more (bacteriostatic test) were not tested for bactericidal effect.

twice daily for seven doses; 4 of the 5 survived. Five rats receiving 4.8 mg./Kg. intramuscularly on the same schedule survived, while 4 of 5 receiving 9.6 mg./Kg. survived seven doses.

In the swimming test for neurotoxicity the subcutaneous dose of colistin sulfate was 18 mg./Kg. twice daily and for polymyxin B sulfate it was 31.5 mg./Kg. A thickening of the subcutaneous tissue at the site of injection (back of the neck) was evident by the third day in the mice receiving polymyxin and by the fifth day in those receiving colistin. Necrosis was observed by the seventh day in some of the mice receiving polymyxin and in one receiving colistin. The test was stopped on the ninth day. No lesions were observed in the control mice. Reactions in the daily swimming test indicated no specific neurotoxicity. By the third day there was a general loss of vigor in both treatment groups and the mice tired quickly and remained afloat with difficulty. The control group showed no impairment in swimming. There was no appreciable change in body weight during the test period, but the animals in the treatment groups had an unthrifty appearance and roughened coats.

PATHOLOGY. The kidneys of 4 rats that died about an hour after receiving 15.1 mg./Kg. intraperitoneally appeared normal grossly. Microscopically, two of the kidneys were congested and three had smaller numbers of cortical tubular segments with somewhat swollen cells. Total kidney damage was very slight, a mild irritative effect.

Three rats that survived 15.1 mg./Kg. intraperitoneally were sacrificed four days later. Grossly there were no abnormal findings in any of the organs and microscopically the kidneys were normal.

The 5 mice that survived 9.6 mg./Kg. intraperitoneally twice daily for seven doses were sacrificed on the afternoon of the fourth day. At autopsy all findings

were normal, except in 2 mice that showed slight, shallow kidney pitting. Microscopically, the three spleens sectioned were normal, the five stomachs showed nothing of note, one liver of three sectioned showed slight hepatic cell vacuolation, and the livers showed mild chronic inflammatory changes (not uncommon in mice). The five kidneys showed a mild renal irritation.

Of 10 mice that survived 9.6 mg./Kg. intramuscularly twice daily for 10 days, 3 had small subcutaneous lumps on the back of the neck. Microscopically, the lumps were found to be subacute abscesses. Otherwise gross examination was normal, including injection sites. Microscopic sections from only 2 of the 10 mice were examined. Mild incidental lesions were noticed in the livers and stomachs, but the kidneys were normal.

The 4 rats surviving 4.8 mg./Kg. intraperitoneally twice daily for seven doses were sacrificed on the afternoon of the fourth day. The kidneys and peritoneums were grossly normal, but the glandular portion of the stomach of 1 rat was markedly mottled gray and red, and that of a second rat was slightly mottled, as seen from the exterior. Internally, all four stomachs appeared damaged, the glandular portion being paler, less rugose, and somewhat roughed. Parts of the damaged area were covered with rather thick crusts of friable yellow exudate. Microscopically, the 2 rats with the most affected stomachs were sectioned. These stomachs were severely and extensively inflamed in their glandular portions, and also showed superficial ulceration in one and deeper ulceration and frank necrosis in the other. The inflammatory cellular exudate strongly involved the mucosa and submucosa. Some edema and slight hemorrhage were present. The livers and kidneys were normal. The spleen contained a slightly abnormal number of polymorphonuclear leukocytes in the pulp, most likely a reaction to the gastric inflammatory process. Sections of the intestines, including some from near the pylorus, showed no inflammation.

The 5 rats surviving 4.8 mg./Kg. intramuscularly twice daily for seven doses and the 4 surviving seven doses of 9.6 mg./Kg. were sacrificed on the afternoon of the fourth day. Grossly, the thigh muscles all were mottled at the injection sites, the effect being slight in the former group and moderate in the latter. The viscera were normal, except for the stomach, but there was much less damage to that organ than was observed after intraperitoneal administration. Three rats from each group were examined microscopically. The thigh muscles had large areas of necrotic fibers and slight edema and hemorrhage. One of the stomachs from the low-dosage group showed moderate acute inflammation under the mucosa near the esophagus, with very little elsewhere. The other two stomachs of this group were negative, as were two of the stomachs from the high-dosage group; one from the latter group showed inflammation, abnormal glands, and superficial ulceration. None of the six livers sectioned showed anything of note. The kidneys of the high-dosage group showed mild irritative changes, definite but not overt. In the low-dosage group these changes were minimal and not definite.

Groups of 5 rats were given oral doses of 55.2 and 27.6 mg./Kg. twice daily until seven doses were given. At the end of the test period the animals were sacrificed and the organs studied. The kidneys, livers, and stomachs appeared normal both grossly and microscopically, except for possibly a trace effect in the stomachs of the high-dosage group.

COLISTINMETHANESULFONATE SODIUM. The preparation of colistinmethanesul-

fonate containing dibucaine hydrochloride had an acute intravenous LD_{50} in mice of 217.7 mg./Kg. and LD_{100} of 289 mg./Kg. The pure colistinmethanesulfonate had an acute intravenous LD_0 in mice of greater than 315 mg./Kg. This derivative was apparently tolerated in mice in dosages many times greater than the sulfate. Pure colistinmethanesulfonate was used exclusively in the chronic toxicity tests in rats.

Five rats received 3.6 mg./Kg. intraperitoneally twice daily for seven days and all survived. Five rats received 35.5 mg./Kg. on a similar schedule; 3 survived only two doses, 1 survived three doses, and 1 survived four doses.

Three rats received 7.1 mg./Kg. intramuscularly twice daily for seven doses and all survived. Three received 35.5 mg./Kg. intramuscularly (5 per cent solution) on a similar schedule; 1 died five hours after the first dose and 2 died after the fourth dose.

PATHOLOGY. The kidneys of the 5 rats surviving seven doses of 3.6 mg./Kg. intraperitoneally were grossly normal, and two of the stomachs had slight irritation. Microscopically, the stomachs and kidneys of all 5 rats were normal.

The 3 rats that died after the second 35.5 mg./Kg. intraperitoneal dose and the 1 that died after the third dose had grossly normal kidneys, but the stomachs showed slight to moderate irregular darkness of the glandular mucosa but no definite lesions. The rat that died after the fourth dose had hemorrhage visible on the wall of the stomach, and the last 2.5 inches of the colon was slightly distended and the wall mottled with hemorrhagic areas. The kidney and leg muscle did not appear grossly damaged. Microscopically, half of the glandular mucosa of the stomach was partly to completely necrotic and the submucosa showed acute inflammatory cellular exudate, hemorrhage, and edema. There was definite kidney damage; about half of the proximal convoluted tubules showed from partial to complete necrosis with some sloughing of the epithelium. The glomeruli appeared undamaged. There was extensive though not massive submucosal hemorrhage of the colon with very little reaction, indicating that the hemorrhage may have been a terminal event.

The 3 rats surviving 7.1 mg./Kg. intramuscularly twice daily for seven doses were sacrificed on the afternoon of the fourth day. There were no gross pathological changes. Microscopically, all three stomachs were normal. The kidney of 1 rat had a single cortical focus of spontaneous nephritis, and the leg muscles of another had scattered necrotic fibers with polymorphonuclear and mononuclear leukocytes in and immediately around them.

The rats that died after receiving from one to four 35.5 mg./Kg. intramuscular doses had from moderate to marked stomach irritation, while the kidneys and muscles at the site of injection appeared normal. Microscopically, there was extensive stomach and kidney damage in the 2 rats examined (died after four doses). Half of the glandular mucosa of the stomach was partly to completely necrotic. The forestomach (squamous epithelium) was essentially unaffected. From one fourth to three fourths of the proximal convoluted tubules of the kidney showed from partial to complete necrosis. There was a moderate amount of necrotizing and inflammatory damage in the leg muscle of 1 rat and a small amount in the other.

Clinical Studies. Individual serum concentrations at two, four, six, and eight hours after single intramuscular injections of 15 and 30 mg. of colistin (as the methanesulfonate) are given in table III. Serum concentrations after doses of 75 and

TABLE III

*Individual Serum Concentrations ($\mu\text{g./ml.}$) of Colistin After a Single Intramuscular Injection of Colistinmethanesulfonate Sodium in Man**

Case no.	Hours after 15 mg. dose†				Case no.	Hours after 30 mg. dose‡			
	2	4	6	8		2	4	6	8
1	0.70	0.25	0.24	0.15	11	1.27	0.69	0.65	0.17
2	0.98	0.43	0.29	0.24	12	1.03	1.09	0.79	0.71
3	0.88	0.46	0.10	0	13	1.16	0.92	0.71	0.77
4	0.78	0.46	0.28	0.16	14	3.12	1.09	0.65	0.41
5	1.16	0.37	0.27	0.23	15	1.79	0.77	0.39	0.29
6	0.88	0.33	0.21	0.21	16	1.52	0.67	0.51	0.36
7	0.75	0.51	0.37	0.21	17	2.11	0.98	0.58	0.58
8	0.83	0.48	0.39	0.29	18	3.27	1.23	0.73	0.51
9	0.88	0.61	0.28	0.13	19	1.27	1.09	0.54	0.22
10	1.70	0.51	0.48	0.19	20	1.60	1.09	0.73	0.81
Average	0.95	0.44	0.29	0.18		1.81	0.96	0.63	0.48

* Control specimens, taken just before medication, were negative.

† Actual assay: 16.8 mg./2 ml.

‡ Actual assay: 34.7 mg./2 ml.

150 mg. of colistin are given in table IV. The high average values obtained two hours after the injection declined rapidly, but the individual values in tables III and IV reveal that the concentrations at each time interval were consistently of the same order of magnitude. No colistin was detected in the blood 24 hours after the 75 mg. dose, but some activity was observed in 8 of 9 subjects 24 hours after the 150 mg. dose. The serum concentrations appeared to increase almost proportionately as the dose was increased. It is interesting to note that regardless of the dose, the rate of declination from a particular blood concentration was the same in several instances. For example, with the 15 mg. dose the average of 0.95 $\mu\text{g./ml.}$ at two hours dropped to 0.18 $\mu\text{g./ml.}$ in another six hours, and after the 75 mg. dose the average of 0.99 $\mu\text{g./ml.}$ at six hours fell to 0.24 $\mu\text{g./ml.}$ in another six hours. The

TABLE IV

*Individual Serum Concentrations ($\mu\text{g./ml.}$) after a Single Intramuscular Injection of Colistinmethanesulfonate Sodium in Man**

Case no.	Hours after 75 mg. dose†						Case no.	Hours after 150 mg. dose‡											
	2	4	6	8	12	24		2	4	6	8	12	14	16	18	20	22	24	
21	2.60	2.40	0.77	0.70	0	0	26	13.00	4.27	2.73	3.40	1.40							0.17
22	2.00	1.20	0.97	0.53	0.25	0	27	6.07	6.67	2.73	1.17	1.40							0.23
23	4.47	2.47	1.40	0.97	0.41	0	28	6.07	6.07	4.07	2.27	0.93							N.S.
24	3.00	1.40	0.47	0.39	0.21	0	29	7.00	3.87	2.47	1.67	1.07							0.09
25	2.67	1.27	1.33	0.61	0.32	0	30	5.80	3.27	1.67	1.33	0.45							0
Ave.	2.96	1.75	0.99	0.64	0.24	0		7.60	4.83	2.73	1.97	1.05							0.12
							31					0.49	0.37	0.27	0.11	0.39	0.14	0.06	
							32					0.57	0.22	0.28	0.14	0.13	0	0.05	
							33					0.65	0.41	0.37	0.39	0.13	0.16	0.13	
							34					0.67	0.34	0.37	0.26	0.30	0.11	0.12	
							35					0.37	0.37	0.27	0.23	0.18	0	0.13	
							Average					0.55	0.32	0.31	0.23	0.23	0.08	0.10	

N.S. = No specimen available for assay.

* Control specimens, taken just before medication, were negative.

† Actual assay: 79.6 mg./2.5 ml.

‡ Actual assay: 150 mg./5 ml.

TABLE V

*Urinary Excretion of Colistin (mg.) after Intramuscular Injections
of Colistinmethanesulfonate Sodium in Man**

Case no.	Hours after 15 mg. dose†					Case no.	Hours after 30 mg. dose†				
	0-2	2-4	4-6	6-8	Total		0-2	2-4	4-6	6-8	Total
1	1.80	0.11	0.15	0.59	2.65	11	4.84	6.64	2.86	1.32	15.66
2	0.45	5.09	0.42	0.04	6.00	12	0.31	5.32	2.06	0.39	8.08
3	1.96	1.66	0.43	0.16	4.21	13	8.04	4.80	2.66	1.46	16.96
4	2.25	1.44	0.81	0.75	5.25	14	8.67	8.80	1.41	1.18	20.06
5	3.21	2.83	3.58	0.55	10.17	15	9.69	1.95	2.96	0.36	14.96
6	3.32	0.44	0.84	0.24	4.86	16	10.00	3.98	2.07	1.77	17.82
7	2.90	6.98	2.60	0.41	12.89	17	0.37	7.70	2.86	1.04	11.97
8	0.32	3.69	0.41	0.46	4.88	18	1.46	1.71	1.61	1.04	5.82
9	3.21	0.79	0.78	0.23	5.01	19	2.71	2.38	0.51	0.55	6.15
10	2.06	1.79	0.77	0.28	4.90	20	8.80	6.00	2.55	2.21	19.56
Average	2.15	2.48	1.08	0.37	6.08		5.49	4.93	2.16	1.13	13.71

* Control specimens, taken just before medication, were negative, except for cases 10 and 14, which gave small zones of partial inhibition that were too indistinct to read.

† See table III for actual assay.

average of 1.81 $\mu\text{g./ml.}$ two hours after the 30 mg. dose fell to 0.63 $\mu\text{g./ml.}$ in another four hours, and the 1.75 $\mu\text{g./ml.}$ four hours after the 75 mg. dose fell to 0.64 $\mu\text{g./ml.}$ in a corresponding length of time. In the amounts used for intramuscular injection, no initial or residual pain was noted at the site of injection.

The urinary excretion of colistin during the eight hour period immediately after the 15 and 30 mg. doses is shown for each subject in table V. It will be noted that 36.2 per cent of the 15 mg. dose and 39.6 per cent of the 30 mg. dose were recovered in the urine during the eight hours after medication. The average urine concentrations of colistin in the time periods of zero to two, two to four, four to six, and six to eight hours after the 15 mg. dose were 14.3, 16.1, 7.0, and 2.7 $\mu\text{g./ml.}$, while after the 30 mg. dose the values were 50.6, 42.8, 20.6, and 7.7 $\mu\text{g./ml.}$, respectively. These values were calculated by dividing the total colistin found in the urine of all 10 subjects of each group by the total volume of urine during each test period.

The results of the multiple dose study of two injections of 15 or 30 mg. of colis-

TABLE VI

*Individual Serum Concentrations ($\mu\text{g./ml.}$) after 2 Intramuscular
Doses of Colistinmethanesulfonate Sodium at 6 Hour Intervals**

Case no.	Dose 1: 15 mg.,† dose 2: 15 mg., hours after each dose			Case no.	Dose 1: 30 mg.,† dose 2: 30 mg., hours after each dose		
	4	2	6		4	2	6
36	0.31	0.88	0.18	36	1.23	1.40	0.41
37	0.48	1.01	0.39	37	0.51	2.13	0.27
38	0.36	0.93	0.20	38	0.81	2.13	0.65
39	0.64	0.14	0.27	39	0.61	1.17	0.39
40	0.51	0.67	0.28	40	0.70	1.79	0.47
Average	0.46	0.98	0.26		0.77	1.73	0.41

* Control specimens, taken just before the first dose, were negative.

† See table III for actual assay.

TABLE VII

Individual Serum and Urine Concentrations ($\mu\text{g./ml.}$) of Colistin after a Single Oral Dose of Colistinmethanesulfonate Sodium in Man

Case no.	Dose, mg.	Blood, hours after dose*				Urine, hours after dose*			
		2	4	6	8	0-2	2-4	4-6	6-8
41	221	0	0	0	0	1.48	2.92	0	0
42	221	0	0	0	0	1.64	1.12	1.52	0.54
43-45	221	0	0	0	0	0	0	0	0
46	750†	0	0	0	0	0.52	0.36	0.26	0
47	750	0	0	0	0	1.95	0	0.17	0
48	750	0.14	0.10	0	0	1.13	2.67	0.90	0
49	750	0.29	0.08	0	0	3.37	1.24	1.37	0.47
50	750	0.31	0.15	0.21	0.11	9.20	4.60	1.37	0.63
51	1500‡	0	0	0	0	0.90	N.S.	0.79	N.S.
52	1500	0.05	0	0	0	2.05	34.00	0.79	0
53	1500	0.08	0.09	0	0	0.99	1.03	0.45	0.72
54	1500	0.70	0.29	0.19	0.19	6.33	8.33	2.93	1.70
55	1500	1.60	0.55	0.40	0.16	30.00	17.33	8.67	3.67

N.S. = No specimen.

* Control specimens of blood and urine, taken just before medication, were negative.

† 726 mg. by actual assay.

‡ 1452 mg. by actual assay.

tin (as the methanesulfonate) six hours apart are given for each subject in table VI. The values found four hours after the first dose agree well with those previously found after single doses (table III), as do the values found two and six hours after the second dose. The latter observation would indicate that with these doses there is no appreciable accumulation in the blood.

As shown in table VII, no colistin serum activity was detected after an oral dose of 221 mg. of colistin as the sodium methanesulfonate; however, some activity was detected in the urine of 2 of the 5 subjects. After the 750 mg. and 1500 mg. doses, serum activity was detected in a majority of the subjects and urinary activity was found in every case. Mild gastrointestinal symptoms consisting of slightly increased bowel activity were observed after the higher doses.

DISCUSSION

Chemical Studies. The infrared spectrum of colistin sulfate agrees substantially with that of colistin hydrochloride reported by Kurihara and Suzuki.¹² The finding of only end absorption in the 210 to 250 $\text{m}\mu$ region of the ultraviolet spectrum is not in agreement with the finding¹² of broad absorption maxima at 225 and 265 $\text{m}\mu$, but does agree with the spectrum given by Koyama¹³ for colistin phosphate. That two fractions were detected in colistin sulfate by descending paper chromatography is not surprising, since crude preparations have been reported to yield from three to five components,¹² and the material used in our work was only about 75 per cent pure.

Colistin has been differentiated from circulin and all the polymyxins except polymyxin E by the amino acid data.

D-Leucine is reported as a constituent of colistin, circulin, and polymyxins A,

D, and E. Although this information is lacking for polymyxins B and C, they may be differentiated from colistin by their phenylalanine constituent and polymyxin D may be differentiated from colistin by its serine moiety. The lack of definite data on L-leucine in circulin and polymyxins A and E led to the finding that the former two had very low L-leucine contents, possibly due to extraneous impurities, while polymyxin E contained about the same as colistin. The low L-leucine values for circulin and polymyxin A may be regarded as reliable in the light of the L-threonine contents, and are evidence that colistin is different from them. Colistin could not be differentiated from polymyxin E by L-leucine content, nor for that matter by the L-threonine contents. The value of 20.9 per cent L-threonine in polymyxin E sulfate appears to be considerably higher than the 9.9 per cent reported in colistin⁹ and the 12.6 per cent found in colistin sulfate lot CB-7710, but it is not significantly higher than the 15.1 per cent in the colistin sulfate master standard; the 18.0 per cent L-threonine in the two lots of polymyxin E base is even closer.

Further work, including chromatography of the intact antibiotics, is necessary to distinguish colistin from polymyxin E.

Toxicity. The acute intraperitoneal LD₅₀ of 20.8 mg. of colistin (sulfate) per Kg. in mice agrees well with the value of 23.0 mg./Kg. reported by Forni and Guidetti.⁵ It may also be compared with the value of 21 mg./Kg. for polymyxin B (sulfate). In the chronic toxicity test none of the 5 mice died from 9.6 mg./Kg. intraperitoneally twice daily for three and one half days. Forni and Guidetti⁵ administered 2 mg./Kg. once daily for 50 days and 16 of 20 mice survived.

The acute intraperitoneal LD₅₀ of 9.6 mg. of colistin (sulfate) per Kg. in rats does not agree with the value of 27.3 mg./Kg. reported by Muggia and Hidalgo,¹⁴ although it is not clear whether they used colistin sulfate or the methanesulfonate.

Mice tolerated injected colistinmethanesulfonate in dosages many times greater than the toxic dosage of the sulfate. This was not observed in rats. Four of 5 rats survived seven intramuscular doses of 9.6 mg. of colistin (as the sulfate) per Kg., while all 3 rats survived after receiving seven doses of 7.1 mg. of colistin (as the methanesulfonate) per Kg., and 1 of 3 died five hours after a single dose of 35.5 mg. of the latter product per Kg. and the other 2 died after four doses.

Pathologically, however, rats did tolerate colistinmethanesulfonate a little better than the sulfate. Animals surviving seven intraperitoneal doses of 4.8 mg. colistin (as the sulfate) showed extensive stomach inflammation, while animals surviving seven doses of 3.6 mg. colistin (as the methanesulfonate) were normal microscopically. One of 3 rats examined microscopically after surviving seven intramuscular doses of 4.8 mg./Kg. (as the sulfate) and 1 of 3 surviving seven doses of 9.6 mg./Kg. showed moderate acute stomach inflammation, while all 3 rats surviving 7.1 mg./Kg. (as the methanesulfonate) had normal stomachs. Rats that died after two to four intraperitoneal doses of 35.5 mg./Kg. (as the methanesulfonate) showed extreme necrosis of the stomach and definite kidney damage.

Blood Concentrations. The 150 mg. intramuscular dose is equivalent to about 2.1 mg./Kg. The highest concentration (two hours after the injection) was 7.6 µg./ml. This agrees proportionately with the 25 µg./ml. reported³ one hour after an intramuscular dose of 5.0 mg./Kg. in normal human adults. The average serum concentrations after the 1500 mg. oral dose were about one half the average values obtained after a 15 mg. intramuscular dose. It appears that approximately 200 times

as much colistinmethanesulfonate must be given orally to attain the serum concentrations obtained from an intramuscular dose. However, the amount of oral colistin may be limited by gastrointestinal side effects.

Urine Concentrations. The 50.6 $\mu\text{g./ml.}$ found in the urine after an intramuscular dose of 30 mg. (0.4 mg./Kg.) is higher proportionately than the value of 300 $\mu\text{g./ml.}$ reported by Forni and Guidetti⁵ for a dose of 5.0 mg./Kg. However, in the first instance the value is for urine pooled for two hours after the dose, while in the latter case it was for urine collected 45 minutes after the dose.

The relationship between serum and urine concentrations would indicate that at any given time the serum concentration of colistin is about one twentieth as great as the urine concentration.

SUMMARY

Colistin resembles the polymyxins and circulin in its structure. Using paper chromatography and amino acid analysis, colistin was differentiated from circulin and polymyxins A, B, C, and D. While some differences were noted between colistin and polymyxin E, their contents of L-leucine and L-threonine were not sufficiently at variance to establish that the two are in fact different.

Colistin sulfate was more active in vitro than polymyxin B sulfate in inhibiting the coli-aerogenes, *Pseudomonas*, *Salmonella*, and *Shigella* organisms. Both were almost inactive against *Proteus*, staphylococci, and streptococci.

The acute toxicity of colistin sulfate in mice was comparable to that of polymyxin B. Colistin sulfate was somewhat more toxic to rats than to mice. Rats that died after an intraperitoneal dose of 15.1 mg./Kg. (slightly in excess of an LD_{50}) had very slight kidney damage, while those surviving had no kidney damage. Rats surviving 4.8 mg. of colistin sulfate per Kg. intraperitoneally twice daily for seven doses showed no toxic effects in the kidney or liver, but the stomachs were severely and extensively inflamed. Twice this dose intramuscularly also produced stomach inflammation, but to a lesser degree. Colistinmethanesulfonate sodium was much less toxic in mice than colistin sulfate, but in rats the former was tolerated only a little better than the latter. In no case was stomach inflammation observed in mice, indicating that this phenomenon in rats is a species characteristic.

Intramuscular injections of colistinmethanesulfonate sodium in doses of from 15 to 150 mg. (0.2 to 2 mg./Kg.) were well tolerated in man and produced peak serum concentrations in proportion to the doses. The serum concentrations diminished relatively rapidly and predictably. After 15 and 30 mg. injections, serum concentrations were detectable for at least eight hours, for 12 hours after a 75 mg. dose, and for 24 hours after a 150 mg. dose. Approximately 40 per cent of the dose was recovered in the urine during eight hours after a single injection of 15 or 30 mg.

Colistinmethanesulfonate is very poorly absorbed from the gastrointestinal tract. No colistin was detected in the serum after an oral dose of 221 mg., but activity was detected in the serum after single doses of 750 and 1500 mg. Only minor gastrointestinal side effects were observed after the doses used. At any given time the concentration of colistin in the urine appeared to be about 20 times that in the serum.

It is estimated that the oral dose of colistinmethanesulfonate must be approximately 200 times the intramuscular dose to obtain comparable serum concentrations.

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Studies on Sodium Colistinmethanesulfonate, Including a Comparative Study of Its Effect on Gram-Negative Organisms

ANCEL BLAUSTEIN

Booth Memorial Hospital, Flushing, N. Y.

Sodium colistinmethanesulfonate was isolated by a Japanese investigator, Koyama et al.¹ It is produced from a soil organism *Aerobacillus colistinus*. It is a polypeptide that is water soluble and heat stable. Guassardo² in Italy as well as numerous other Italian and French investigators have studied exhaustively the use of this antibiotic in all phases of clinical medicine. On this continent I have had the opportunity to repeat some of the laboratory aspects of this investigation. The study comprised toxicity evaluation, blood level studies, and in vitro comparison with 17 other antibiotics in relation to gram-negative organisms.

TOXICITY STUDIES

Ten healthy subjects received 83.32 mg. of sodium colistinmethanesulfonate intramuscularly four times a day for five days. Ten subjects received 166 $\frac{1}{3}$ mg. of sodium colistinmethanesulfonate four times each day intramuscularly. Each of the 20 subjects had the following battery of studies performed initially, on the third, and on the sixth day: hemoglobin, hematocrit, white blood count, differential blood studies, blood sugar, alkaline phosphatase, total protein and albumin-globulin ratio, serum bilirubin, blood urea nitrogen, and a routine urinalysis.

None of the subjects found the individual injections painful; however, the frequency of the injection was annoying. There were no subjective or objective symptoms following any of the total of injections. No significant alterations in blood count, differential, urinalysis, or blood chemistries were noted. Two subjects showed a slight rise in alkaline phosphatase levels, but these never exceeded the normal range.

IN VITRO COMPARISON WITH OTHER ANTIBIOTICS IN RELATION TO GRAM-NEGATIVE ORGANISMS

In this study sensitivity discs were supplied in 50 units and 300 units. Eighty gram-negative organisms, from hospitalized patients, were studied for sensitivity response to sodium colistinmethanesulfonate, polymyxin B, novobiocin, neomycin, ristocetin, sulfamethoxypyridazine, furazolidone, kanamycin, streptomycin, tetracycline, oxytetracycline, nitrofurantoin, sulfisoxazole, Ro5-0810/1, sulfadimethoxine, and vancomycin. A high degree of sensitivity was demonstrated by sodium colistinmethanesulfonate against resistant strains of *Bacillus pyocyaneus* and intermediate forms of the coli-aerogenes group. The drug was at least equally effective against *Escherichia coli*, *Escherichia freundii*, *Aerobacter aerogenes*, *Bacillus ani-*

tratum, *Paracolobactrum aerogenoides*, *Paracolobactrum coliforme*, and *Paracolobactrum intermedium*. In some cases a higher degree of sensitivity was noted in otherwise resistant variants of *E. coli* and *Paracolobactrum coliforme*. It is ineffective in sensitivity studies against most *Proteus* strains.

BLOOD LEVELS

Guassardo in 1958 in his excellent report on sodium colistinmethanesulfonate reported on blood level studies with this drug. He reported cumulative effects of three times daily doses (50,000 units/Kg.). After the first injection, he noted blood levels of 100 to 200 units/ml. until the third and fourth hour, after which it slowly decreased until the ninth and tenth hour when it was completely absent from the blood. In the subsequent days of therapy blood levels rose to 400 to 600 units/ml.

Two subjects, one of whom received 83.32 mg. of sodium colistinmethanesulfonate four times a day and another 166.33 mg. four times a day, had blood samples drawn after two, four, six, and eight hours on the first day, as well as two hours after the first injection on the fifth day. All levels obtained were greater than those for the standard curve, even at dilutions of 1:10 of serum. This would, it appears, compare favorably with the results reported by Guassardo.

SUMMARY

It would appear that sodium colistinmethanesulfonate is nontoxic and non-painful on injection. High blood levels can be quickly attained and the antibiotic is exceedingly effective in the management of a large number of gram-negative infections.

ACKNOWLEDGMENT

I wish to express my appreciation to the Warner-Lambert Research Institute which supplied the sodium colistinmethanesulfonate.

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In Vitro Sensitivity of *Pseudomonads* from Burned Patients to Colistin Sulfate

CHARLES D. GRABER, WILFRED T. TUMBUSCH, AND EDWARD H. VOGEL, JR.

*U. S. Army Surgical Research Unit, Brooke Army Medical Center,
Fort Sam Houston, Texas*

The causative agent of bacteremia in burned patients hospitalized at the U.S. Army Surgical Research Unit, Brooke Army Medical Center, has shifted in the last six years from preponderantly staphylococcal to *Pseudomonas* organisms.¹ Table I documents this transition for the period 1953 to October 1, 1959. The disparity in this ratio is especially great for the current year. Utilizing available indicated antibiotics, there has been but one survivor in these burned patients in the last three years where *Pseudomonas aeruginosa* septicemia has occurred as a complication. The increased incidence of *Pseudomonas* bacteremias has stimulated our interest in the Japanese antibiotic colistin sulfate (Coly-mycin S*) and its reputed bactericidal action for this organism. To date, sodium colistinmethanesulfonate (Coly-mycin M†), the parentally injectable form of colistin sulfate, has not been accorded a suitable clinical trial, although 10,000,000 units of this drug were given to a burned patient with *Pseudomonas* bacteremia during the two days preceding his death. At autopsy, anatomical lesions incompatible with life were demonstrated so that sodium colistinmethanesulfonate had no assessable effect although terminal and postmortem blood cultures were negative.

The testing described in this paper has been limited to evaluating tube sensitivity of nosocomial and non-nosocomial strains of *Pseudomonas aeruginosa* to colistin sulfate. The nosocomial organisms were recovered from the blood stream, urine, and skin of fatally burned patients hospitalized during the past two years at this unit. All strains had shown considerable insensitivity to neomycin, polymyxin, kanamycin, and broad-spectrum antibiotics. Serologic analysis with unabsorbed O antibody made to the National Institute of Health strain antigens had shown these strains to have different somatic antigens. Phage typing with undifferentiated and undesignated *Ps. aeruginosa* phages also showed differences in phage pattern. The non-nosocomial pseudomonads were obtained from another laboratory and were mainly fecal in origin. These organisms were not serologically differentiated.

RESULTS

Table II records the sensitivity of the nosocomial pseudomonads to the four antibiotics. In the case of two drugs, neomycin and kanamycin, the bactericidal level exceeded realistic, achievable plasma level of these drugs. To a somewhat less extent, this is true of the polymyxin B although most of the 18 strains were sensitive in the range 12.5 to 25 µg./ml. The exquisite sensitivity of *Pseudomonas*

* The trade name of Warner-Chilcott Laboratories for colistin sulfate is Coly-mycin S.

† The trade name of Warner-Chilcott Laboratories for sodium colistinmethanesulfonate is Coly-mycin M.

TABLE I

Increase in Incidence of Pseudomonas over Staphylococcal Bacteremias in Burn Patients
US Army Surgical Research Unit—1953–1959

	1953–1954	1955–1956	1957–1958	1959 (1 Oct)
Total deaths (septicemic)	17	22	23	14
Deaths associated with <i>Staph. aureus</i> (pure or mixed)	10 (58%)	13 (59%)	8 (35%)	2 (14.3%)
Deaths associated with <i>Pseudomonas aeruginosa</i> (pure or mixed)	8 (47%)	13 (59%)	15 (65%)	9 (64.4%)

TABLE II

Comparison of Tube Sensitivities of Nosocomial Pseudomonads to 4 Antibiotics

<i>Pseudomonas</i>	Source*	Neomycin, μg./ml.	Kanamycin, μg./ml.	Polymyxin B, μg./ml.	Colistin sulfate μg./ml.
62	S	>25	>25	25	3.12
4-9-3	B	>25	>25	25	0.78
4-3-1	B	12.5–25	>25	12.5–25	1.50
65	S	>25	>25	12.5–25	0.78
4-10-1	B	>25	>25	12.5–25	0.78
4-3-11	B	>25	>25	12.5–25	1.50
4-8-4	U	>25	>25	12.5–25	3.12
12-26-24	B	>25	>25	12.5–25	3.12
2-18-1	B	>25	>25	12.5–25	0.78
2-17-8	B	>25	>25	12.5–25	1.50
11-24-9	B,U	>25	>25	25	0.78
2-10-5	B	>25	>25	12.5–25	3.12
4-2-8	B	>25	>25	12.5–25	1.5
12-25-3	B	>25	>25	12.5–25	1.5
66	S	>25	>25	12.5–25	1.5
7-28-3	B	>25	>25	25	1.5
7-28-8	B	12.5–25	12.5–25	12.5–25	3.12
7-28-10	B	>25	>25	12.5–25	0.78

* B = burn; S = skin; U = urine.

TABLE III

Sensitivity of Non-Nosocomial Pseudomonads to 4 Antibiotics

<i>Pseudomonas</i> no.	Source	Neomycin, μg./ml.	Kanamycin, μg./ml.	Polymyxin B, μg./ml.	Colistin sulfate, μg./ml.
1	Feces	3.12	.78	6.25	3.12
2	Ear	6.25	6.25	12.5	0.78
3	Feces	3.12	3.12	6.25	1.5
4	Feces	6.25	6.25	6.25	0.78
5	Pleural fluid	6.25	6.25	12.5	1.5
6	Feces	6.25	6.25	12.5	1.5
7	Vagina	3.12	3.12	25.0	0.78
8	Rectum	1.5	1.5	6.25	0.78
9	Feces	3.12	0.39	6.25	<0.39
10	Feces	3.12	6.25	12.5	3.12
11	Feces	6.25	6.25	12.5	0.78
12	Feces	6.25	6.25	12.5	0.78
13	Feces	3.12	3.12	3.12	0.78

TABLE IV

In Vitro Development of Antibiotic Resistance of Pseudomonads to Colistin Sulfate

	Titer change after 6 serial transfers			
	None	Twofold	Threefold	Fourfold
<i>Ps. aeruginosa</i> Strains				
12 (Nosocomial)	6	3	2	1
13 (Non-nosocomial)	3	6	2	2
Total	9	9	4	3

to colistin sulfate is demonstrated by the fact that only five of the strains required as much as 3.12 µg./ml. of this drug for bactericidal activity.

Thirteen non-nosocomial pseudomonads obtained from the Fourth United States Army Area Laboratory, Fort Sam Houston, Texas, demonstrated also great sensitivity of bactericidal character to colistin sulfate. From the data recorded in table III, only two of the strains required as much as 3.12 µg./ml. for bactericidal inhibition while eight of the strains were killed by 0.78 µg./ml. or less of the drug. The non-nosocomial pseudomonads appear somewhat more sensitive than the hospital strains to the other three drugs; however, in only 1 case is there evidence of better sensitivity to any of these drugs than to colistin sulfate.

The emergence of resistance of *Ps. aeruginosa* to colistin sulfate was of interest and 12 of the nosocomial and 13 of the non-nosocomial pseudomonads were tested to determine if resistance was enhanced by sublethal serial transfer. Six serial transfers in noninhibiting concentrations of colistin sulfate showed only 3 of the 25 strains (table IV) demonstrating more than a threefold titer increase in antibiotic needed for bactericidal activity. Only one nosocomial and two fecal strains displayed this acquisition of resistance. Since non-paired testing was done, it is possible that this represents only mechanical error.

SUMMARY

In summation, the in vitro testing described strongly suggests that colistin sulfate is worthy of a trial in the management of *Pseudomonas* septicemia complicating severe burns.

ACKNOWLEDGMENT

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Clinical and Laboratory Observations on the Use of Colistin in Infections by Gram-Negative Bacilli

WILLIAM R. MCCABE, GEORGE GEE JACKSON, AND VERA M. KOZIJ

*University of Illinois Research and Educational Hospitals and Department of Medicine,
University of Illinois, College of Medicine, Chicago, Ill.*

Colistin is a new antibacterial polypeptide that has been recovered from *Aerobacillus colinistus*.¹ It has been in clinical use in Japan and Europe and was recently introduced for investigation in this country. The spectrum of antibacterial activity includes predominantly gram-negative genera. In vitro studies have demonstrated antibacterial activity against most genera of the enterobacteriaceae, including a number of strains of *Pseudomonas aeruginosa*.¹⁻³ This paper reports our data from in vitro studies with colistin and the results of treatment with colistin among patients with urinary tract and other infections produced by gram-negative bacteria.

METHODS AND MATERIALS

Laboratory. The antibiotic sensitivity of strains of various gram-negative bacteria to colistin was determined by serial twofold tube dilutions. The tube containing the least amount of antibiotic that had no visible growth after 24 hours' incubation was considered the minimal inhibitory concentration. The minimal bactericidal concentration was considered the lowest concentration of antibiotic that inhibited visible growth at 24 hours and prevented regrowth of 0.05 ml. of inhibited culture on antibiotic-free medium.

Colistin levels in serum and urine specimens from patients were determined by serial twofold dilutions of the specimen and the addition of 0.5 ml. of 1:10,000 dilution (10^4 — 10^5 bacteria) of an 18 hour broth culture of a standard strain of *Escherichia coli* (0.22 K⁻ H-4). The sensitivity of the inoculum to a colistin standard was made at the time of determination of antibiotic levels of each group of serum and urine specimens. The antibiotic concentration was calculated as the product of the reciprocal dilution of serum or urine that inhibited growth and the minimal inhibitory concentration of the test strain. All values are expressed as $\mu\text{g.}$ of colistin base calculated on the basis of 30 units/ $\mu\text{g.}$ of base.

Clonal variation in colistin sensitivity of the standard strain was tested by the pour plate method. Pour plates were made containing varying antibiotic concentrations and 10^9 , 10^8 , 10^4 , and 10^3 bacteria. Bacterial colonies were counted after 24 hours' incubation and the proportion of bacteria resistant to varying antibiotic concentrations was determined.

Clinical. Colistin was given to patients with infections caused by gram-negative bacteria. These largely consisted of patients with chronic bacteriuria, septicemia, and soft-tissue or other infections caused by *Ps. aeruginosa*. The infecting organism was obtained and its sensitivity to colistin determined. Blood and urine samples

These studies were supported in part by a grant from Warner-Lambert Research Institute, Morris Plains, New Jersey.

TABLE I

Sensitivity to Colistin Among 66 Strains of Various Gram-Negative Bacteria Isolated from Clinical Infections

Bacterial species	Minimal inhibitory concentration, µg./ml.			
	<0.1	0.1-0.9	1.0-10	>10
<i>E. coli</i>	5	12	18	3
<i>Paracolon</i> species	1	0	2	1
<i>A. aerogenes</i>	0	0	4	6
<i>Pseudomonas</i>	1	1	2	2
<i>Proteus</i> species	0	1	0	7

were obtained at times of high (two hours) and low (six hours) activity after intramuscular injection of 33.3 mg. of colistin. Patients with urinary tract infections received colistin for five days; the duration of treatment being dictated by an investigational protocol. The length of treatment among the other patients was determined by clinical evaluation. Serial urinalysis, hemograms, and studies of renal and hepatic function were obtained in all patients.

RESULTS

The sensitivity to colistin of 66 strains of various gram-negative bacteria isolated from patients treated with colistin alone or in conjunction with other antibiotics is shown in table I. Among 38 strains of *E. coli*, 17, or 45 per cent, were inhibited by less than 1 µg./ml. of colistin, and all but three, or 92 per cent, were inhibited by 10 µg. or less; 8 per cent were resistant to 10 µg./ml. Among the remaining 18 strains of *E. coli*, 12 were inhibited by less than 5 µg./ml., making 73 per cent of the total strains sensitive to this concentration.

Four strains of paracolon bacillus were greatly different in their susceptibility to colistin; one was extremely sensitive, one extremely resistant, and two were inhibited by 1 to 10 µg./ml. Among 10 strains of *Aerobacter aerogenes*, none was inhibited by less than 1 µg./ml. and six were resistant to concentrations of colistin in excess of 10 µg./ml. *Ps. aeruginosa* was isolated from 6 patients. Two of these strains were sensitive to less than 1 µg./ml., two were resistant to greater than 10 µg./ml., and the remaining two had intermediate sensitivity to colistin. All but one

TABLE II

Comparative Inhibitory Activity by Weight of Colistin, Tetracycline, Chloramphenicol, and Nitrofurantoin for Some Gram-Negative Bacteria Recovered from Clinical Infections

Drug	No. strains tested	Inhibited by <5.0 µg./ml.	
		Number	Per cent
Colistin	38	28	73
Tetracycline	38	25	66
Chloramphenicol	38	16	45
Nitrofurantoin	38	2	5

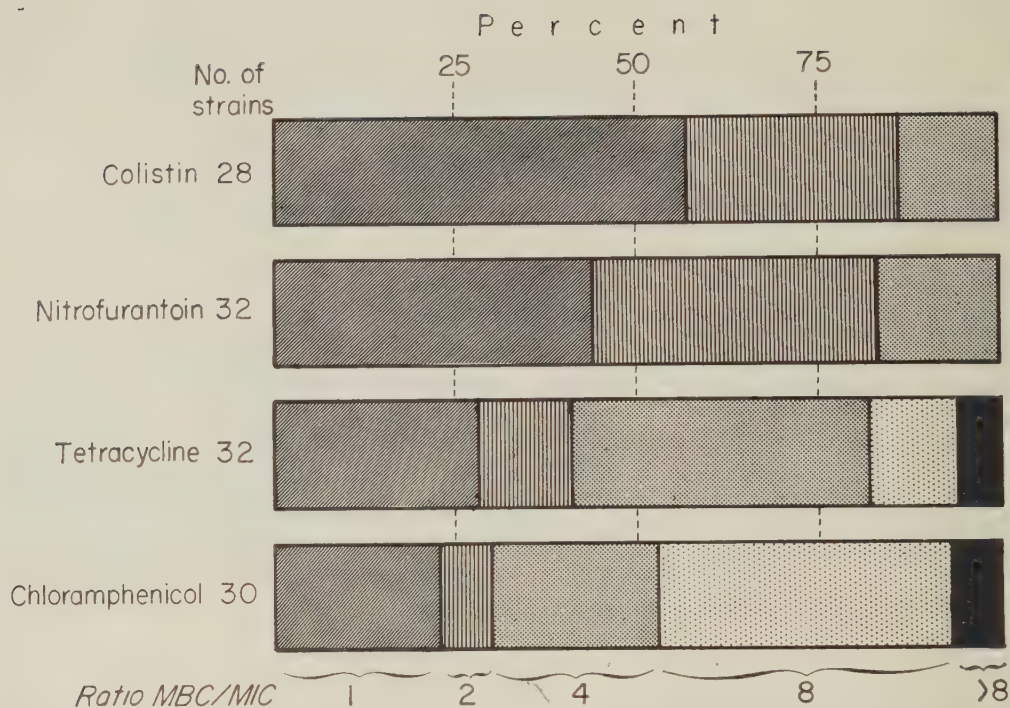


FIG. 1. In vitro bactericidal activity among four antibacterial substances: MBC/MIC = minimum bactericidal concentration/minimum inhibitory concentration.

of eight strains of *Proteus* species were resistant to more than 10 $\mu\text{g./ml.}$ of colistin; it was inhibited by less than 1 $\mu\text{g./ml.}$

The sensitivity of most of these 66 strains from clinical infections also was determined for tetracycline, chloramphenicol, and nitrofurantoin. The number of strains that was inhibited by 5 $\mu\text{g./ml.}$ of colistin (table II) was greater than with the same concentration of any of the other three agents. This difference was not statistically significant for the tetracycline, but the effectiveness of colistin was very significant in comparison with that of chloramphenicol ($p = <0.01$) and nitrofurantoin ($p = <0.001$). Among the strains of *E. coli* and *A. aerogenes*, tetracycline was more effective than colistin on a weight basis, but chloramphenicol and nitrofurantoin were uniformly less effective than colistin in the proportion of each species inhibited by 5 $\mu\text{g./ml.}$ of the respective drugs. Only colistin among these antibacterial agents inhibited the strains of *Ps. aeruginosa* at concentrations less than 10 $\mu\text{g./ml.}$

In figure 1 the in vitro bactericidal activity of colistin is compared with those of nitrofurantoin, tetracycline, and chloramphenicol. The results are expressed as the ratio of the minimal bactericidal concentration to the minimal inhibitory concentration. For 57 per cent of the strains of various gram-negative species, the minimal bactericidal and inhibitory concentrations of colistin were the same; for 29 per cent the former was twofold greater; and for 14 per cent the former was four times the latter. In none of the observations did this ratio exceed 4. A similar pattern of bactericidal activity was observed with nitrofurantoin. Tetracycline and especially chloramphenicol were much less bactericidal at or near the inhibitory concentration. For 59 per cent of the strains tested with tetracycline, the minimal

bactericidal concentration was fourfold or more greater than the minimal inhibitory concentration; it was eightfold or more for 18 per cent of strains. With chlor-
amphenicol, the minimal bactericidal concentration for 70 per cent of the strains was fourfold or more greater than the minimal inhibitory concentration and for nearly one half, it was eight or more times the latter.

One peculiarity of colistin that was encountered in the laboratory studies was the occasional occurrence of a tube with turbidity from bacterial growth in the midst of clear tubes with inhibited growth from greater or lesser concentrations of colistin. This was shown to be a characteristic of several strains and not due to contamination. To clarify this phenomenon, the clonal variation in sensitivity to colistin among cells in a culture of the standard test strain of *E. coli* was studied. Figure 2 shows the number of bacteria required for the detection of one resistant colony at increasing concentrations of colistin. All of the organisms inoculated into the medium grew at a concentration of 0.26 µg./ml. A twofold increase in colistin inhibited 90 per cent of the cells with which the medium was inoculated. A second twofold increase in colistin inhibited all but 1 in 1000 bacteria; 1 in 100,000 cells was resistant to an eightfold increase in drug concentration, and one in a billion organisms grew in a 16- or 32-fold increase in colistin, i.e., 4.2 or 8.4 µg./ml.

To examine whether the bacterial growth that occurred in sporadic tubes emerged from the selection of resistant cells, subcultures of the parent and aberrant strain were studied. The inhibitory concentration of colistin for the aberrant strain was found to be 64 times that of the parent strain. The clonal distribution of the two strains by resistance to increasing concentrations of colistin showed that all of the organisms in the aberrant culture grew at a drug concentration in which only 1 in 100 million organisms from the parent culture grew. Thus, on the basis of these studies, facultative single step mutation seemed to be the most likely explanation for the sporadic growth of drug-resistant cultures in the laboratory.

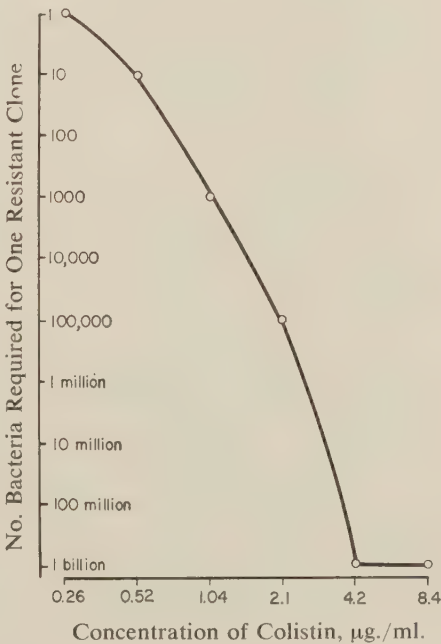


FIG. 2. The clonal variation in sensitivity to colistin is indicated.

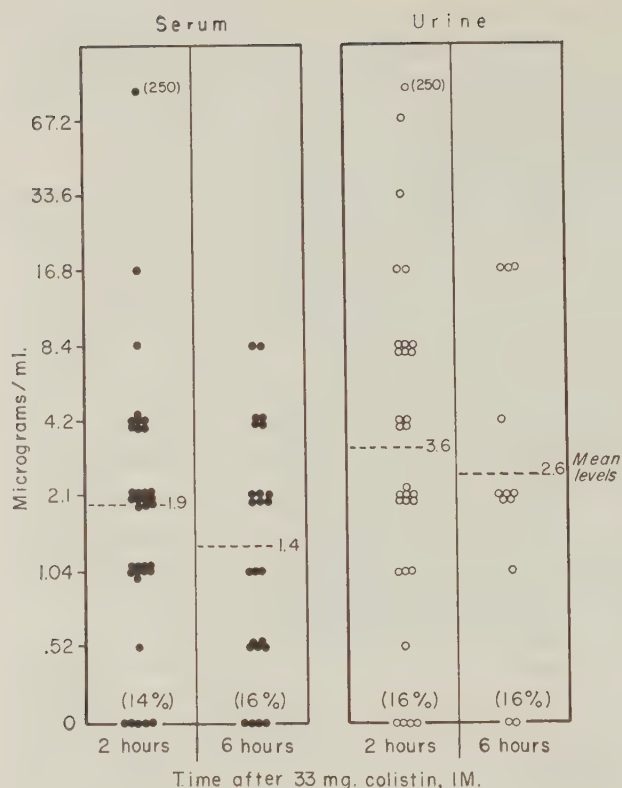


FIG. 3. Concentration of colistin in serum and urine two and six hours after 33 mg. of colistin.

Figure 3 shows the colistin levels observed in 103 specimens of serum or urine collected at times judged to represent peak and trough levels after the intramuscular injection of 33 mg. of colistinmethanesulfonate. More than 84 per cent of the specimens of serum or urine had antibiotic activity. The geometric mean level in the serum two hours after colistin administration was 1.9 $\mu\text{g./ml.}$ This was five times the inhibitory concentration of the standard strain of *E. coli*. Six hours after the dose, the mean level was 1.4 $\mu\text{g./ml.}$ or, on the average, an inhibitory concentration at a 1:1.8 dilution of serum. The urine concentrations were slightly higher; the mean at two hours was 3.6 $\mu\text{g./ml.}$ and at six hours it was 2.6 $\mu\text{g./ml.}$

Fifty-three patients were treated with colistin. Nineteen of them received colistin in combination with other antibiotics and are included only in the tabulation of drug toxicity. The results of treatment in 21 patients with chronic urinary tract infections are given in table III. Five patients were excluded because of failure to complete treatment or incorrect initial diagnosis. The results of an immediate post-treatment urine culture and another obtained two or three months later are compared with those in a similar previous group of patients treated with intravenous nitrofurantoin. The results are comparable or slightly better than was observed with nitrofurantoin. Eighty-one per cent of the patients had sterile urine cultures immediately after five days of colistin treatment. Four, or 19 per cent, continued to have significant bacteriuria. Two, or 13 per cent, of the patients with complete follow-up had had a relapse of the bacteriuria two or three months after colistin treatment. Of 16 patients in whom complete information is available, 6, or 38 per cent, failed to attain either immediate cure or else relapsed within three months

of treatment. In comparison, 50 per cent of the previous group treated with nitrofurantoin had failed or relapsed.

Seven patients who had gram-negative septicemia and 1 patient with bacterial endocarditis due to *E. coli* were treated with colistin in a dosage of 33 mg. every six hours. One patient each with bacteremia with *E. coli* or *Proteus mirabilis* and 1 patient with the typical clinical syndrome of gram-negative septicemia but negative blood cultures responded clinically and bacteriologically to colistin. Three patients with bacteremia due respectively to *E. coli*, *A. aerogenes*, and *Ps. aeruginosa* failed clinically and bacteriologically to respond to colistin. Two of the latter patients also had pyelonephritis, which failed to respond to colistin, and are included in the previous tabulations. One other patient had several blood cultures that contained *E. coli* and *Bacteroides* species. He had no clinical response to seven days of colistin treatment or to a subsequent 14 days of tetracycline therapy. No positive blood cultures were obtained, however, after the beginning of colistin therapy, and he had spontaneous defervescence two weeks after the discontinuation of tetracycline. The patient with *E. coli* endocarditis had been ill for five months and had not been cured by treatment with other antibiotics. Colistin was efficacious only during its administration, and bacteriological relapse occurred promptly after discontinuation on three occasions. One patient was treated for pneumonia associated with *Ps. aeruginosa*. There was no clinical response and a fatal meningitis with *Pseudomonas* occurred during colistin administration. Four patients had postoperative wound infections caused by *Ps. aeruginosa*. These all responded by defervescence and prompt healing.

In 1 patient who had transient clearing of bacteriuria and then prompt relapse after colistin treatment for chronic pyelonephritis, the development of resistance was observed. The strain of *E. coli* isolated from the urine after treatment was 32 times more resistant to colistin than the strain isolated before treatment, and serological typing of the strains showed them to be the same type (0.18a, 18c, B:21 H:7).

Although 53 per cent of the 53 patients had some side reaction to colistin, nearly all of the reactions were very mild. Paresthesias were the most common and were observed in 13 patients. These were of moderate severity in 3 patients but in only

TABLE III

Treatment of Chronic Urinary Tract Infections with Colistin in Comparison with a Previous Group Treated with Intravenous Nitrofurantoin

Results	Colistin		Intravenous nitrofurantoin*	
	No. of patients	Per cent†	No. of patients	Per cent
Initial				
Success	17	81	9	75
Failure	4	19	3	25
Late†				
Cure	10	63	6	50
Relapse or initial failure	6	37	6	50

* Reference 6.

† Late results refer only to patients re-examined four to six weeks after treatment.

1 was it necessary to discontinue treatment because of the patient's complaints. Of 10 patients who had nausea, vomiting, diarrhea, or abdominal cramping, all but 1 were receiving other oral antibiotics. Neutropenia and granulocytopenia were observed in 3 patients. In 1, a patient with hemolytic anemia and hemoglobinuria, spontaneous neutropenia had been observed previously and a subsequent course of colistin caused no difficulty. In a second patient, the total leukocyte count decreased after four days of treatment from 9250 to 3000/cu. mm. without a distinct change in the percentage of granulocytes. Several other antibiotics were given simultaneously with colistin. The count promptly returned to normal limits after discontinuation of treatment. The third patient had transient anemia, thrombocytopenia, and granulocytopenia three days after institution of treatment with ristocetin and colistin for a fecal fistula.

Urticaria limited to a tattoo on the forearm and reproduced by another injection of colistin was observed. The observation also was confirmed by intradermal injection of colistin in normal skin with failure to produce a wheal, whereas intradermal colistin given in the tattoo site produced a wheal within five minutes.

No evidence of renal or hepatic toxicity was observed in any of the 53 patients given colistin.

DISCUSSION

Colistin is reported to be a complex polypeptide with a molecular weight of about 1200.¹ It is relatively stable and not readily inactivated in body fluids. On a weight basis, colistin has a high degree of antibacterial activity; 1/30 μ g. (1 unit) of base inhibits the growth of a standard strain of *E. coli*. The active base forms stable salts of which two, the sulfate and sodium methanesulfonate, have been available for study.

The antibacterial spectrum of colistin is remarkably similar to that of polymyxin. Excepting members of the *Proteus* species, some strains of all the common gram-negative bacilli are inhibited by a few μ g./ml. As is the case with polymyxin, strains of *Ps. aeruginosa* may be sensitive to colistin.^{1,3} Use of colistin in patients with wound infections caused by *Ps. aeruginosa* provides some in vivo support for its effectiveness, which was found by European investigators.⁴

Another significant property of colistin observed in the laboratory is its high degree of bactericidal activity. The bactericidal concentration of colistin was the same or only twofold greater than the minimal inhibitory concentration of 86 per cent of the strains of enterobacteriaceae that we studied. This was distinctly higher than that demonstrated by antibiotics that are commonly used for systemic treatment of infections caused by gram-negative bacteria.

In spite of the bactericidal activity of colistin, laboratory observations suggested that some organisms escaped inhibition and gave rise to drug-resistant strains. The study of clonal variation in colistin sensitivity revealed the occurrence of one cell in a billion that was 8 to 16 times more resistant to colistin than 90 per cent of the bacteria in the same culture. The observation suggests the occurrence of facultative single step mutation of *E. coli* to drug resistance of this magnitude. If this is the case, one could anticipate the emergence of resistant strains in some patients under treatment. Indeed, this was documented in 1 case. Such a phenomenon also

seems the most likely explanation for the sporadic growth of bacteria inoculated into tubes containing more than the inhibitory concentration of colistin. The kinetics of growth for the surviving cells are not known. They are all important, however, since at least 10 per cent of bacteria may be viable at the minimal inhibitory concentration and nevertheless fail to emerge as drug-resistant progeny. Mutants that give rise to resistant strains must not only survive but proliferate at an uninhibited rate. If facultative single step mutation and emergence of drug-resistant strains are shown to occur with many strains of bacteria, it may be that use of a second antibiotic with colistin should be recommended.

Colistin in an active form is absorbed poorly and irregularly from the gastrointestinal tract but after intramuscular injection significant antibacterial activity appears in the blood within 30 minutes and reaches a peak at two to three hours after injection.² The height and duration of the plasma concentration are directly proportional to the size of the dose.¹ Active drug is excreted promptly in the urine and in our observations was concentrated twofold. The mean plasma concentration decreased only 25 per cent between two and six hours after the dose; thus the rate of excretion was less than the normal rate of glomerular filtration, although the polypeptide is not strongly bound to plasma proteins.¹

Antibacterial activity was demonstrable in more than 84 per cent of serum and urine specimens collected from patients at times when peak and trough levels were expected. The mean peak serum level from a 33 mg. dose every six hours was approximately the same as the mean inhibitory concentration for the bacterial strains recovered from these patients.

The results obtained with colistin in the treatment of chronic urinary tract infection compared favorably with those obtained in a previous study with intravenous nitrofurantoin. They also confirm the results of Robecchi and Favro,⁵ who treated 29 patients with *E. coli* urological infections for three to five days with 62 per cent cure or good improvement. Final evaluation of treatment in our patients was not made until repeat urine cultures had been obtained at least two months after the completion of treatment. It is well known that symptomatic improvement and even urine cultures in the immediate post-treatment period are inadequate to determine eradication of urinary tract infection.^{6,7} Similarly, colistin appeared valuable in the treatment of wound infections caused by *Ps. aeruginosa* but failed in the treatment of one half the patients with gram-negative septicemia. These results also are comparable to those of others.⁸

Side effects from colistin were frequent and usually consisted of digital and circumoral paresthesias. They were rarely of significant consequence. Pain on injection was infrequent, but the preparations we used always contained 2 mg. of dibucaine per dose. Sensitization of the skin by a prior tattoo with production of urticaria from colistin was definite but it is unexplained and was clinically insignificant. The three instances of leukopenia observed were believed to be unrelated to colistin, and 1 of these patients was re-treated with colistin without difficulty.

Several similarities may be noted between colistin and polymyxin. Both are polypeptides with a similar antibacterial spectrum, especially in the demonstration of antibacterial activity against *Ps. aeruginosa*; both exhibit a high degree of bactericidal activity; there is marked cross resistance among bacterial strains;^{1,3} and both cause paresthesias during treatment. Thus, the mechanism of action is probably the same

but colistin appears to be free from the renal toxicity that is characteristic of polymyxin, but also to have a less uniform effect against *Ps. aeruginosa*.

SUMMARY AND CONCLUSION

Colistin is a new polypeptide antibiotic with a high degree of bactericidal activity against many strains of different species among the enterobacteriaceae, including *Ps. aeruginosa*. Both its spectrum and specific activity on a weight basis closely resemble those of polymyxin B. Individual cells among one billion *E. coli* were shown to differ in sensitivity by 8- to 16-fold and facultative single step drug-resistant mutation is suggested.

After intramuscular injection of 33 mg. of colistin, appreciable antibacterial activity was present in 84 per cent of serum and urine specimens collected at two and six hours after the dose. The mean serum level was 1.9 and 1.4 $\mu\text{g./ml.}$ at two and six hours respectively and the mean urine level at the same time was 3.6 and 2.6 $\mu\text{g./ml.}$ respectively.

A significant clinical and bacteriological response from treatment with colistin was observed in 62 per cent of urinary tract infections, four wound infections, and 3 of 6 cases of septicemia caused by gram-negative bacilli. One patient died with *Ps. aeruginosa* meningitis. A colistin-resistant strain of *E. coli* emerged in one instance.

Minor side effects from treatment were common but no clinically serious reactions were believed to be caused by colistin, and in contrast to polymyxin, renal irritation was absent.

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Colistin: Some Preliminary Laboratory and Clinical Observations in Specific Gastroenteritis in Infants and Children

SYDNEY ROSS, JOSE R. PUIG, AND EDMUND A. ZAREMBA

Research Foundation of Children's Hospital, Washington, D. C.

Colistin (Coly-mycin*), an antibiotic with a polypeptide chemical configuration, has been found to possess a high degree of bactericidal specificity against many gram-negative organisms in vitro.¹ The bacterial spectrum of colistin approximates that of polymyxin B very closely, even though chemically colistin differs from polymyxin B in not containing phenylalanine in the polypeptide linkage. Like polymyxin B, colistin has the important asset of producing no significant number of resistant strains in vitro in originally susceptible organisms.

Isolated from *Bacillus colistinus* in Japan in 1950 by Koyama et al¹ and subsequently tested clinically in Europe,^{2,4} colistin has not been used in the United States until the present time. The unusual feature of colistin has been the virtual lack of reported toxicity even though it is a polypeptide, since, up to now, the polypeptide antibiotics (viz., polymyxin, bacitracin) have been uniformly characterized by their potential toxicity, a factor that has delimited their widespread use. The desirability of having a nontoxic antibiotic that is effective against certain gram-negative organisms, such as the coliform bacteria and *Pseudomonas* species, has been manifest for some time. Hence the current interest in this antibiotic.

The purpose of the present study was to determine the in vitro sensitivities to colistin of recent clinical isolates of certain gram-negative bacilli (primarily enteric organisms) and simultaneously to compare them with polymyxin B, to determine the degree of absorption of colistin† in infants and children after both intramuscular and oral administration of a given dose, to assess the degree of toxicity of colistin both clinically and from laboratory studies, and to treat a group of infants and children with specific gastroenteritis due to microorganisms susceptible to colistin in vitro, in order to determine its clinical efficacy in this group of infections.

ANTIMICROBIAL ACTIVITY OF COLISTIN IN VITRO

The in vitro sensitivities to colistin of 104 recent clinical isolates, including *Pseudomonas* species, *Shigella*, *Salmonella*, and pathogenic *Escherichia coli*, and of 18 stock cultures of *Shigella* were determined employing the serial tube dilution method in tryptose phosphate broth. These sensitivities were compared with polymyxin B and are presented in table I.

As will be noted, the in vitro activity against *Pseudomonas* species with colistin ranged between 0.95 and 7.8 µg./ml., with the majority of strains (42 of 44) being

* The trade name of Warner-Chilcott Laboratories for colistin is Coly-mycin. The drug employed in this study was generously supplied by Dr. John Pepper.

† The potency of colistin is currently being expressed in mg. One mg. of the pure colistin base contains 30,000 units. The salt forms of colistin employed in the present study contained 20,000 units/mg. of colistin base.

TABLE I

Comparison of the *in Vitro* Sensitivities of 122 Strains to Colistin and Polymyxin B

Strain	No. of strains	Colistin*		Polymyxin B†	
		Range, $\mu\text{g./ml.}$	Majority of strains sensitive to $\mu\text{g./ml.}$	Range, $\mu\text{g./ml.}$	Majority of strains sensitive to $\mu\text{g./ml.}$
<i>Pseudomonas</i> species	44	0.95–7.8	3.9	0.95–7.8	1.9
<i>Salmonella</i>	16	0.48–0.95	0.48	0.24–0.95	0.48
<i>Shigella</i> (recent isolates)	17	<0.12–0.24	<0.12	<0.12–0.24	<0.12
<i>Shigella</i> (stock cultures)	18	<0.12–0.24	<0.12	<0.12–0.24	<0.12
Pathogenic <i>E. coli</i>	26	<0.12–1.9	0.48	<0.12–1.9	0.48

* Colistin sulfate potency—1 $\mu\text{g.}$ = 20 units.† Polymyxin B potency—1 $\mu\text{g.}$ = 7.0 units.

inhibited by 3.9 $\mu\text{g./ml.}$ or less. The similarity of *in vitro* activity of colistin and polymyxin B against these strains of *Pseudomonas* is readily apparent (fig. 1). The slight differences can hardly be considered significant in view of the inherent errors in the method of sensitivity determinations.⁵

Sixteen strains of *Salmonella* species including two strains of *Salmonella typhosa* were inhibited by concentrations of colistin ranging from 0.48 to 0.95 $\mu\text{g./ml.}$, with the majority of strains being sensitive to 0.48 $\mu\text{g./ml.}$ As with *Pseudomonas*, the close parallel in antimicrobial activity between colistin and polymyxin B was again clearly manifest.

Against 17 recent clinical isolates of *Shigella*, the minimal inhibitory concentration of colistin varied from less than 0.12 to 0.24 $\mu\text{g./ml.}$, with the majority of strains being inhibited by less than 0.12 $\mu\text{g./ml.}$ The same *in vitro* activity was obtained with stock strains as with recent isolates. Again, the close parallel to polymyxin B was evident.

With 26 strains of pathogenic *E. coli*, the range of inhibition with colistin varied from less than 0.12 to 1.9 $\mu\text{g./ml.}$, with the majority of strains being sensitive to 0.48 $\mu\text{g./ml.}$ or less. There was no significant difference in sensitivity between the various serotypes of pathogenic *E. coli*. The close parallel in activity of colistin and polymyxin B against the latter species is graphically illustrated in figure 2.

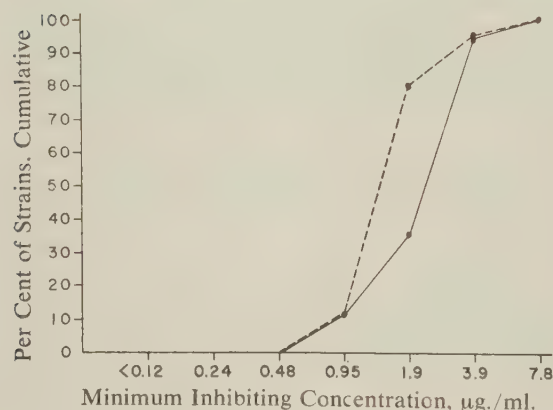
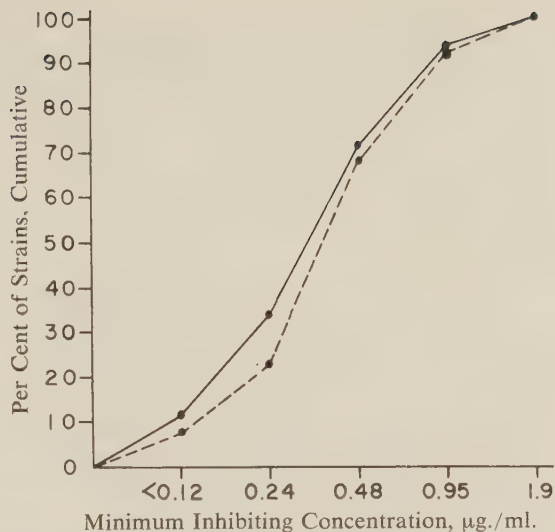


FIG. 1. Comparison between the sensitivities of 44 strains of *Pseudomonas* species to colistin and polymyxin B. — colistin; — — — polymyxin B.

FIG. 2. Comparison between the sensitivities of 26 strains of pathogenic *E. coli* to colistin and polymyxin B. — colistin; --- polymyxin B.



The results of these sensitivity tests thus emphasize the fact that enteric bacteria such as *Salmonella*, *Shigella*, and pathogenic *E. coli* are readily inhibited by colistin in vitro. *Pseudomonas* species were also sensitive to colistin but to a lesser degree. These data also point up the close similarity in antimicrobial activity of colistin and polymyxin B against the species tested. Thus, of the 122 organisms tested, not a single instance was observed where an organism was resistant to polymyxin B and sensitive to colistin, or vice versa. The sensitivities ran parallel with both drugs except for an occasional one or two tube difference, which is within the inherent error of the serial tube dilution method.⁵

Of singular interest was the one strain of pathogenic *E. coli* that, prior to colistin therapy, was readily inhibited by both colistin and polymyxin B in a concentration of 0.48 µg./ml. During therapy, however, the organism became increasingly resistant so that 15.6 µg./ml. of colistin was required for inhibition; simultaneously, the organism had also become resistant to polymyxin B (15.6 µg./ml.), suggesting cross resistance between colistin and polymyxin B.

ABSORPTION OF COLISTIN

Intramuscular Administration. For intramuscular use, sodium colistinmethanesulfonate was available as a sterile powder in multiple dose vials. The powder was dissolved in 5 ml. of sterile distilled water and the appropriate dose was injected deep intramuscularly into the buttocks.

A single intramuscular dose of sodium colistinmethanesulfonate of 2.5 mg./Kg. body weight was given to a group of 4 children ranging in weight from 20 to 40 Kg. Blood was drawn for assay at 1, 3, 6, 12, and 24 hours. Serum concentrations of colistin were determined by a cylinder plate method using *Brucella broncho-septica* ATCC 4617 as the test organism.

The resulting colistin assays in µg./ml. were averaged and are presented in figure 3. As will be noted, a peak concentration of 5 µg./ml. was achieved after one hour followed by a rapid drop to 1.4 µg./ml. after three hours; subsequent

levels were 0.53 $\mu\text{g./ml.}$ after six hours and 0.07 $\mu\text{g./ml.}$ at 12 hours; there was no detectable drug after 24 hours. The rapidity of excretion of colistin in children is noteworthy when compared to that in adults, since in the latter, with comparable dosage, the antibiotic is eliminated slowly, maintaining a therapeutic serum level up to 12 hours.⁶

A suggested intramuscular dose of 7.5 mg./Kg. daily of colistin in a six hour divided dosage schedule should suffice in the majority of susceptible systemic infections.

Oral Administration. Colistin sulfate powder was available for oral administration in the present study. When reconstituted with water, the powder dissolved readily and the resulting solution contained approximately 40 mg./5 ml.

Similar to other polypeptide antibiotics, such as polymyxin B and bacitracin, the absorption by the gastrointestinal tract of orally administered colistin is negligible in adults and older children. In small infants, however, there may be some absorption of colistin orally.⁶ Our own preliminary data would suggest that the absorption of the drug from the gastrointestinal tract in infants was spotty and unpredictable; hence one would rely only on orally administered colistin in infections confined to the gastrointestinal tract, whereas in sytemic infections the intramuscular route would be used.

In the pediatric age group, an oral dose of 15 mg./Kg. daily in an eight hour divided dosage schedule would be ample.

TOXICITY OF COLISTIN

Antibiotics having a polypeptide linkage, such as tyrothricin, bacitracin, and polymyxin B, are known to be unduly nephrotoxic and cephalotoxic. In view of this, the patients in our series were carefully observed clinically and pertinent laboratory examinations were performed during the course of colistin therapy including frequent hemograms, urinalyses, blood urea nitrogen determinations, thymol turbidity, and cephalin flocculation assays.

It was our general impression that colistin, in spite of being a polypeptide, was relatively nontoxic after both oral and intramuscular administration with the dosages employed in the present series. As previously noted, the dosage orally was 7.5 to 15 mg./Kg. daily and the intramuscular dosage was 2.5 to 5 mg./Kg. daily.

The only indication of potential nephrotoxicity occurred in infants less than 7 months of age. There were 11 infants aged 7 months or younger in our series. Of

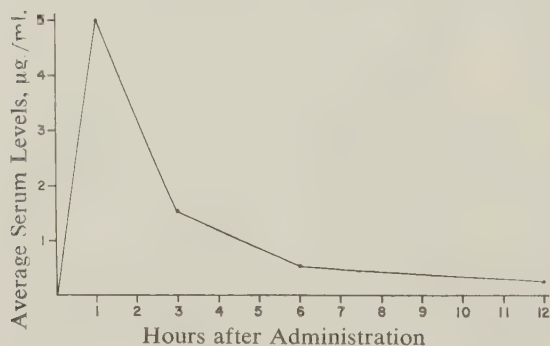


FIG. 3. Serum levels of colistin (average) after a single intramuscular dose of 2.5 mg./Kg. of body weight.

TABLE II

*Highest Blood Urea Nitrogen Elevation after Initiation of Colistin Therapy
in 7 Infants less than 7 Months of Age*

Patient	Age	Wt., Kg.	Dosage of colistin, mg./Kg./day	Highest blood urea nitrogen after therapy started, mg./100 ml.
D. W.	2 mo.	3.0	2.5 intramuscular	17
G. M.	2 mo.	4.2	2.5 intramuscular	25
R. A.	7 mo.	6.1	2.5 intramuscular	25
F. F.	6 wk.	2.4	2.5 intramuscular	21
D. C.	4 mo.	4.8	2.5 intramuscular	19
J. C.	4 mo.	4.1	15 (oral)	25
A. G.	6 wk.	3.8	15 (oral)	19

these, 8 received 2.5 mg./Kg. intramuscularly daily, with 5 infants (62 per cent) showing a mild blood urea nitrogen increase ranging from 17 to 25 mg./100 ml.; 2 of the 3 infants (66 per cent) less than 7 months of age who received 15 mg./Kg. daily orally also had a slight increase in blood urea nitrogen ranging from 19 to 25 mg./100 ml. (table II). None of the remaining 26 children in the present series showed any blood urea nitrogen elevation during courses of therapy ranging from 7 to 16 days. Urinary findings such as white cells, casts, and albuminuria were noticeably absent in every case; this included the 7 infants previously described who had slight azotemia. There was no evidence of hematopoietic depression or liver function impairment nor was any eosinophilia observed. Similarly no drug fever, malaise, or gastrointestinal disturbances were in evidence in our series.

This lack of toxicity was in striking contrast to results previously observed by us when polymyxin B, a closely related polypeptide, was employed intramuscularly in a group of infants and children.⁷ The marked disparity in toxicity between the two drugs after intramuscular administration of comparable doses is clearly evident from table III. Thus, the admonition against using polymyxin B except in severe susceptible infections does not appear to obtain with colistin. Colistin may be regarded as the first representative of a group of polypeptide antibiotics (which are products of

TABLE III

*Comparison of the Incidence of Toxic Reactions after Intramuscular Administration
of Colistin and Polymyxin B*

Type of toxic reaction	Incidence of toxicity, per cent	
	Colistin, intramuscular (2.5-5.0 mg./Kg./day), 24 cases	Polymyxin B, intramuscular (3.0 mg./Kg./day), 12 cases
Fever	0	66
Malaise and anorexia	0	50
Increased nitrogen retention	21	41
Albuminuria	0	33
White blood cells and/or casts in urine	0	75
Leukocytosis	0	50
Eosinophilia	0	16

bacilli) of sufficiently low toxicity to be administered parenterally without significant untoward reactions. One would advise close surveillance for potential nephrotoxicity only in the very young.

CLINICAL EXPERIENCE WITH COLISTIN IN SPECIFIC GASTROENTERITIS

During the past eight months colistin has been routinely employed in the Diarrhea Ward at Children's Hospital in all cases of specific gastroenteritis. A brief summary of these clinical trials is presented.

Colistin in Shigella Enteritis. There were 7 cases of *Shigella* dysentery treated with colistin, the ages of the children ranging from 6 months to 4 years. All 7 cases were caused by type B. During treatment, stool cultures were obtained at periodic intervals until the patients were discharged from the hospital.

Colistin was administered by the oral route in 5 cases, while the intramuscular route was employed in the remaining 2 patients. The oral dosage was 15 mg./Kg. body weight and the intramuscular dosage was 7.5 mg./Kg. daily, both being given in an eight hour divided dosage schedule. The duration of treatment ranged from 9 to 16 days with an average of 12.5 days/patient. The in vitro sensitivities of the seven strains of *Shigella* encountered in this series all were less than 0.12 µg./ml.

If it can be assumed that the rapidity of disappearance of the pathogen from the stool is a rough therapeutic index of the effectiveness of drug therapy in *Shigella* dysentery, it must be concluded that colistin is not so effective as other modalities of therapy we have previously employed. In only 1 of these 7 cases did the stool become promptly negative for *Shigella* organisms within 24 hours. In the remaining 6 patients, positive stool cultures were still encountered for periods ranging from 5 to 13 days after colistin therapy had been initiated; the average time required for sterilization of the bowel was 6.6 days. No differences were noted between patients receiving the drug orally and intramuscularly.

In a series of 304 cases of *Shigella* dysentery treated at Children's Hospital over a seven year period,⁸ it was found that other antibiotics produced a much more dramatic response than colistin, with a prompt disappearance of the *Shigella* organism after initiation of therapy (table IV). The average interval for achieving a negative stool culture with chloramphenicol therapy was 1.3 days; with chlortetra-

TABLE IV
Comparison of the Elapsed Time (Average Days) Required to Obtain Negative Stool Cultures in the Treatment of Shigella Enteritis with Various Antibiotics Including Colistin

Drug	No. of patients with <i>Shigella</i> enteritis	Time required to obtain negative stool culture (average, days)
Colistin	7	6.6
Polymyxin B (oral)	16	1.9
Streptomycin (oral)	34	1.6
Chloramphenicol	153	1.2
Oxytetracycline	39	1.4
Chlortetracycline	46	1.6
Tetracycline	26	1.2

TABLE V

*Comparison of the Efficacy of Colistin and Polymyxin B
in the Treatment of Shigella Enteritis*

	Colistin	Polymyxin B
Number of <i>Shigella</i> cases	7	16
In vitro sensitivities, $\mu\text{g./ml.}$	<0.12	<0.12
Dosage, mg./Kg./day	15 (oral) 7.5 (intramuscular)	18 (oral)
Duration of therapy (average days)	12.5	10.6
No. of days required for negative stool culture	6.6	1.9

cycline, 1.6 days; with oxytetracycline, 1.4 days; with tetracycline, 1.2 days; with oral streptomycin, 1.6 days; and with oral polymyxin B, 1.9 days. This is to be contrasted with the previously noted 6.6 days for colistin. The relative lack of effectiveness of colistin is particularly noteworthy when the drug is compared with polymyxin B, since the latter was found to be quite effective in the treatment of *Shigella* dysentery (table V). It is difficult to explain the somewhat disappointing performance of colistin in *Shigella* enteritis, since all the strains were extremely sensitive to the drug in vitro and the organisms showed no increase in colistin resistance during the course of therapy.

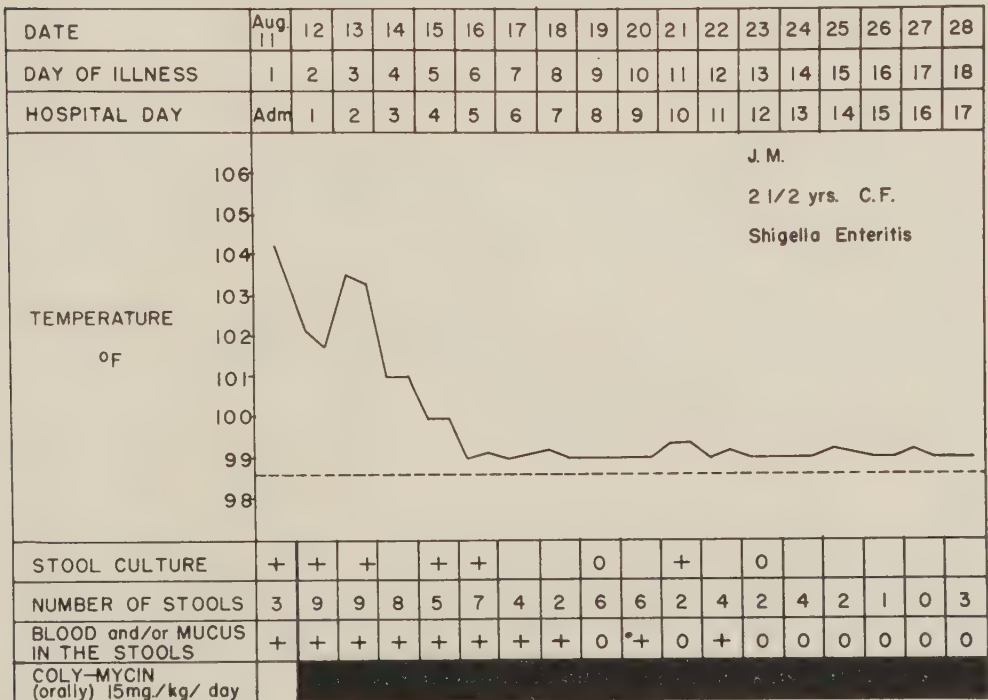


FIG. 4. *Shigella* enteritis treated with colistin. This case illustrates the relatively poor response to colistin therapy encountered in the majority of children with *Shigella* enteritis. A positive stool culture was still obtained 10 days after therapy was started and clinically the response was also belated, with bloody diarrhea continuing for an unduly long period. In the chart colistin is mentioned as Coly-mycin.

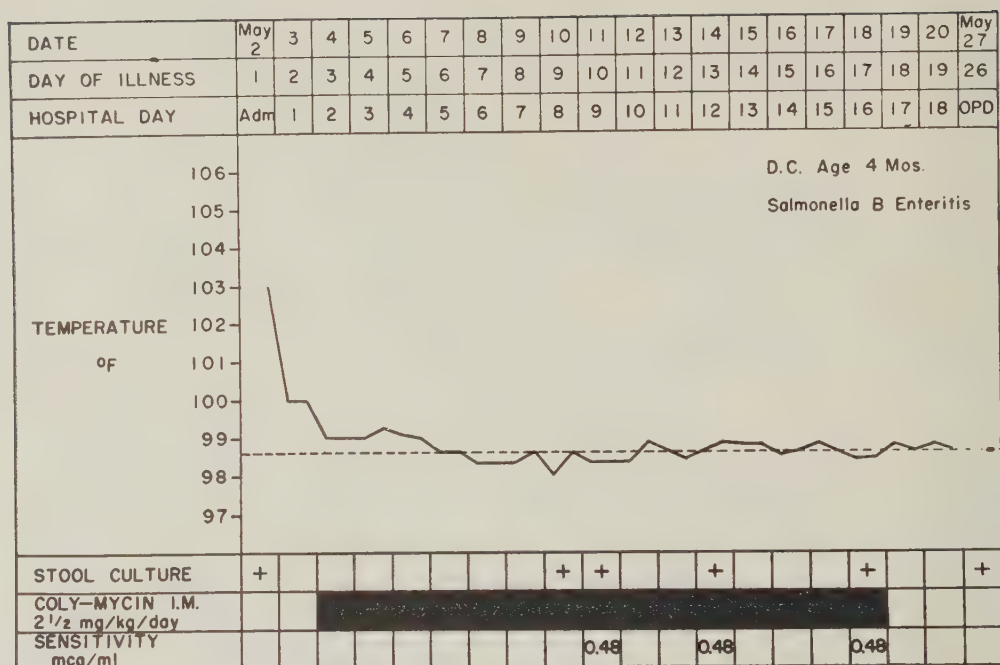


FIG. 5. This case is illustrative of *Salmonella* enteritis treated with colistin intramuscularly. The stool culture remained positive throughout the entire 15 day course of therapy, although clinically the infant responded well. No increase in colistin resistance was encountered here. Other patients with *Salmonella* enteritis responded more rapidly bacteriologically when the drug was given orally rather than intramuscularly. In the chart colistin is called Coly-mycin.

Along with the nondramatic bacteriological response there was a rather slow clinical improvement in 4 of the 7 children treated with colistin. The temperature usually decreased by lysis and the stool consistency gradually improved. One patient still had blood and mucus in the stools even after nine days of therapy (fig. 4). This is in contrast to the rapidity of clinical improvement observed with the other antibiotics previously noted, where the clinical subsidence of the disease was commensurate with the favorable effect observed bacteriologically.

Colistin in Salmonellosis. Ten infants and children with salmonellosis were treated with colistin. In 9 of the cases, *Salmonella* B was cultured from the stool, while the tenth case grew out *Salmonella* E. The colistin sensitivities of these organisms varied from 0.48 to 0.95 $\mu\text{g./ml.}$

Colistin was given orally to 6 of the patients in a dosage of 7.5 mg./Kg. daily in an eight hour divided dosage regimen. The duration of therapy ranged from 13 to 20 days.

The results of treatment were varied. In 5 of the 10 cases, the stool culture became negative within 4 to 10 days after therapy was initiated and remained negative during the remainder of the hospital stay. All 5 of these patients had received the drug orally. The other 5 cases were frank treatment failures with repeat stool cultures remaining positive throughout the entire hospital course, which ranged from 20 to 27 days (fig. 5). Four of these unsuccessfully treated children had received colistin intramuscularly, while the fifth was treated orally. There was no increase in colistin resistance during therapy in the latter group. Thus the failure of colistin can hardly be explained on the basis of acquired resistance of the organism.

In summary, the efficacy of colistin in salmonellosis is still somewhat indeterminate from these preliminary results. It would appear that oral administration of the drug offers greater promise in *Salmonella* enteritis than does the intramuscular mode of administration; in the present series, all 4 patients receiving the drug intramuscularly were frank failures, whereas in 5 of the 6 cases treated orally, the results were modestly good. In those cases of salmonellosis where the organism is not limited to the intestinal tract, however, a combination of oral and intramuscular administration would probably be preferable.

The equivocal results in salmonellosis with colistin were not entirely unexpected. During the past 12 years, we have treated more than 150 cases of *Salmonella* enteritis at Children's Hospital. All the broad-spectrum antibiotics, including chloramphenicol, oxytetracycline, chlortetracycline, and tetracycline, as well as oral streptomycin, polymyxin B, neomycin, and humycin, were employed either alone or in combination. It may be generally stated that an inhibitory effect on both the *Salmonella* organisms and the normal stool flora was observed during the period of administration of each drug. However, in approximately 70 per cent of the cases, the stool either remained persistently positive during the course of drug administration or became free of the pathogen shortly after initiation of therapy only to show a bacteriological relapse after discontinuation of therapy. It might be added that there was no attendant clinical exacerbation associated with these cultural relapses. By and large the various strains of *Salmonella* were sensitive in vitro to all of the antibiotics previously noted. However, after bacteriological reversal, the organism showed no demonstrable increase in resistance when compared with the in vitro sensitivity before treatment was started. Thus, except for transitory sterilization of the bowel of the pathogen during the treatment phase and for varying intervals thereafter, none of the antibiotics employed was capable of effecting a permanent cure bacteriologically in the majority of cases.

In the present series, colistin appeared to compare favorably with the more efficacious modalities of therapy we have employed thus far in the treatment of salmonellosis. Further trials are indicated, however, before any definitive conclusions are warranted.

Colistin in Pathogenic E. coli Gastroenteritis. Eleven infants and children (8 of whom were less than 1 year of age) were diagnosed as having gastroenteritis due to pathogenic *E. coli* and were treated with colistin. The serotypes of *E. coli* included 0-111 (3 cases), 0-125 (2 cases), 0-126 (2 cases), 0-127 (2 cases), 0-55 (1 case), and 0-124 (1 case). The colistin sensitivities of these organisms varied from less than 0.12 to 0.95 $\mu\text{g./ml.}$

Nine of the 11 patients received colistin orally in dosages ranging from 7.5 to 15 mg./Kg. daily in an eight hour divided dosage schedule; the remaining 2 patients were treated with intramuscular colistin, 2.5 mg./Kg. daily. The duration of therapy ranged from 6 to 14 days.

The results of colistin therapy were generally good; usually, within a short time after initiation of therapy, the stool cultures became negative and remained free of pathogens during the remainder of the hospital course. Clinically, the improvement was commensurate with the salutary effect observed bacteriologically.

One of the 11 cases, however, was regarded as a frank treatment failure. This infant's gastroenteritis was caused by serotype *E. coli* 0-126 with an initial colistin

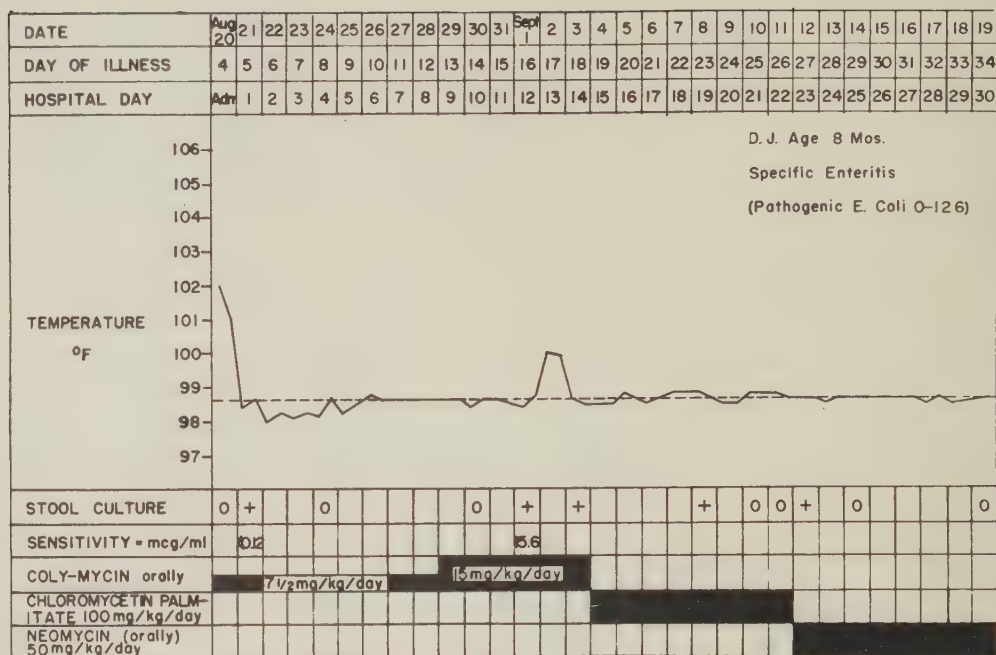


FIG. 6. This 8 month old infant with gastroenteritis due to pathogenic *E. coli* (0-126) was the only treatment failure out of 11 patients with this disease entity. The stool culture was still positive after 14 days of oral colistin, with the organism showing a fivefold increase in resistance within 12 days. Chloramphenicol palmitate also failed to eradicate the organism. Stool cultures remained persistently positive until neomycin was started. Colistin is mentioned in the chart as Coly-mycin.

sensitivity of 0.48 $\mu\text{g.}/\text{ml}$. In spite of rather intensive oral colistin therapy, the stool cultures remained persistently positive, and within 12 days after initiation of therapy, the organism was found to be inhibited by 15.6 $\mu\text{g.}/\text{ml}$., a fivefold increase in resistance (fig. 6).

In summary, it was our impression that colistin affected the course of the disease favorably both bacteriologically and clinically in all cases but 1.

Colistin in Typhoid Fever. Because of the demonstrated susceptibility of *Sal. typhosa* in vitro to colistin, it was considered of interest to assess the efficacy of this drug in typhoid fever. Two patients with typhoid fever were treated with colistin. A summary of 1 of the cases is presented below.

A. E., a 4 year old Negro girl, was admitted to Children's Hospital, Aug. 21, 1959, with the complaints of fever and abdominal pain of six days' duration. The child had been in good health until the onset of the present illness, at which time she began to run a temperature elevation and complained of intermittent abdominal pain. The fever continued to range between 102 and 104 F., and two days after the onset, she was seen by her local physician. Three consecutive daily intramuscular injections of procaine penicillin were given, but there was no perceptible clinical improvement or defervescence of temperature. Hospitalization was therefore advised. Past and family history were noncontributory.

Physical examination revealed a moderately ill child who appeared in no acute distress. The temperature was 104.2 F.; the pulse rate, 120; and the respiratory rate, 28. Pertinent findings included a mildly injected pharynx, a slight degree of epigastric tenderness, and minimal tenderness in both flank areas. Physical examination was otherwise negative.

Laboratory results showed a hemoglobin of 11.0 Gm., 4500 white blood cells with 65 per cent neutrophils, 14 per cent band forms, 17 per cent lymphocytes, 3 per cent monocytes, and

1 per cent eosinophils. The urinalysis was negative. Blood urea nitrogen was 8.0 mg./100 ml. A roentgenogram of the chest was normal. The agglutination test for *Sal. typhosa* (0 antigen) was positive in a dilution of 1:80; five days later the titer had risen to 1:640. A blood culture taken on admission was positive for *Sal. typhosa*; a stool culture taken four days after hospital admission was also positive for *Sal. typhosa*. The colistin sensitivity of the organism isolated from the blood culture was 0.95 µg./ml.

Colistin therapy was started on the fourth day after admission. The dosage was 60 mg. every eight hours intramuscularly (7.5 mg./Kg. body weight per day). Blood cultures became sterile after the second day on colistin and remained negative on three consecutive cultures taken over the course of the next seven days. A stool culture obtained five days after the start of therapy was negative, and, except for one positive culture two days later, all the subsequent stool cultures were negative. Periodic urinalyses during colistin therapy showed no albuminuria or white cells. Similarly there was no increase in blood urea nitrogen.

In spite of the favorable bacteriological effects of colistin therapy, no clinical improvement was perceptible. The temperature continued to fluctuate between 101 and 105 F. daily, and the child remained moderately ill (fig. 7).

In view of the relatively disappointing clinical response to colistin, the drug was discontinued after nine days and chloramphenicol was substituted; the latter drug was given as the succinate in a dosage of 1 Gm. every 12 hours intramuscularly. Within 48 hours, there was rapid clinical improvement and the child remained afebrile during the remaining six days of her hospital stay. She was discharged on Sept. 10, 1959, after 21 days in the hospital.

A second patient with typhoid fever was started on colistin therapy 15 days after the onset of illness. A low-grade temperature persisted during the next four days

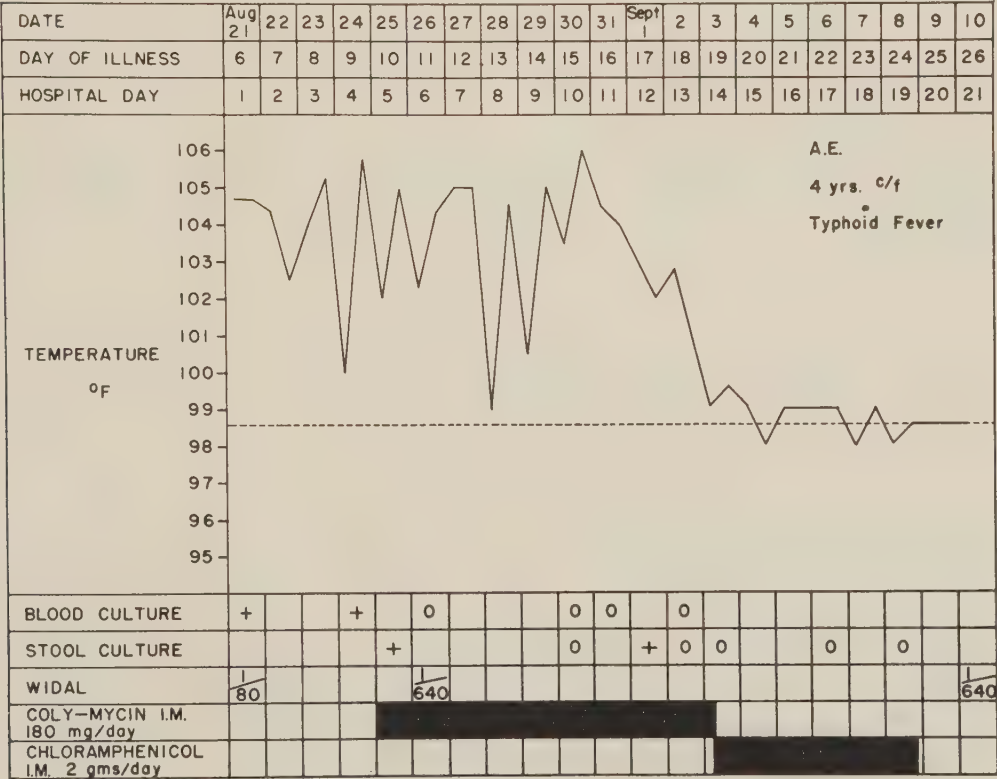


FIG. 7. Case of typhoid fever treated with colistin. In this instance, stool cultures five days after start of colistin therapy and subsequently, with the exception of culture seven days after instigation of therapy, were negative. Blood cultures became sterile after the second day. No clinical improvement was perceptible, however. In the chart colistin is called Coly-mycin.

followed by a gradual defervescence thereafter. Blood and stool cultures were negative both before and during colistin therapy; a positive Widal titer of 1:1280 confirmed the diagnosis. In this case, it was felt that the drug was initiated too late in the course of illness to calibrate any part played by colistin in altering the course of the disease.

Although additional clinical experience would be required before any definitive statements are possible, one does get the preliminary impression that colistin will not be an effective agent in the management of typhoid fever.

SUMMARY AND CONCLUSIONS

1. Colistin is a polypeptide antibiotic, which was originally isolated in Japan and recently introduced into the United States.

2. The in vitro sensitivities to colistin of 104 recent clinical isolates, including *Pseudomonas* species, *Shigella*, *Salmonella*, and pathogenic *E. coli*, and of 18 stock strains of *Shigella* were determined and compared to those to polymyxin B. It was found that these species were readily inhibited by colistin in vitro and that there was a close similarity in antimicrobial activity between colistin and polymyxin B against the species tested.

3. Colistin was readily absorbed and rapidly excreted after intramuscular administration but unpredictably or negligibly absorbed after oral administration in infants and children.

4. Colistin, in sharp contrast to other polypeptide antibiotics, proved to be relatively free of toxicity after both oral and intramuscular administration in the pediatric age group.

5. Colistin received a clinical trial in a group of children with specific gastroenteritis. Generally, the results were good in pathogenic *E. coli* gastroenteritis, fair in *Salmonella* enteritis, somewhat disappointing in *Shigella* enteritis, and poor in typhoid fever.

ACKNOWLEDGMENTS

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Treatment of Acute Diarrheas of Infancy with Oral Colistin Sulfate

JOSEPH GREENGARD AND ANGEL F. ALISEDA

*Children's Division, Cook County Hospital, and Department of Pediatrics,
University of Illinois College of Medicine, Chicago, Ill.*

Infantile diarrheal disturbances continue to pose a serious problem in hospital practice. Many of these cases are due to enteral infection, and an antibiotic that is active in the lumen of the bowel, is free of deleterious side effects, and has a wide range of potency against gram-negative organisms could be of great help in the management of these cases. Colistin is a new antibiotic that gives promise of playing such a role. It is a complex polypeptide isolated in Japan from the micro-organism *Aerobacillus colistinus*. It is effective against a wide range of gram-negative organisms and exhibits moderate activity against some gram-positive organisms as well. It is available in two salt forms: colistin sulfate and sodium colistimethanesulfonate.

REVIEW OF LITERATURE

There are no reports of the use of this drug in diarrhea of infancy in the American literature. Guassardo¹ reported clinical and experimental research on colistin in 1958. He found that, in addition to powerful bactericidal and bacteriostatic activity against gram-negative strains in the intestinal flora, the antibiotic also possessed activity against some *Candida* strains. He felt that it might be used in other disturbances in infancy and childhood, specifically urinary tract infections and pertussis. Good results were also obtained in meningitis, peritonitis, and otitis. In the latter group the drug was used intramuscularly in doses of 50,000 units/Kg. of body weight per day. He found, however, when given orally to children in doses of 100,000 to 200,000 units/Kg. of body weight, blood levels reach 80 to 120 units/ml. Increasing the oral dose to 180,000 to 200,000 units/Kg./day yielded levels similar to those attained by intramuscular injection. He treated 48 infants ranging from 10 days to 5 months of age with diarrhea, none of whom had improved on dietary therapy. He obtained rapid improvement after colistin was administered; all were cured after five to six days, and there was relapse in only 3 cases. A second series of 74 infants ranging from 15 days to 2 years with gastroenteritis were treated. Generally improvement was very rapid, with subsidence of fever, toxemia, and vomiting within 24 to 48 hours and resumption of normal diet by the eighth day. In 2 cases there was no improvement and the infants died. In 4 cases there was mild relapse after the drug was stopped, but recovery after a second course of therapy.

Sacrez et al² observed 196 infants infected with *Escherichia coli* 0111 B4 in the period from February to the beginning of August, 1957. There were 22 deaths. Neomycin and framycetin were used in the first 59 infants and seemed progressively ineffective. The next 52 cases were treated with polymyxin B. Clinical improvement was rapid in the mild cases. There were 4 deaths. Stools were sterilized in 2 to 6 days in 5 healthy carriers. Colistin was used in 36 cases, most of them a few weeks

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old in dosages of 125,000 units/Kg. body weight. Duration of therapy averaged 8 to 10 days, the extremes being 6 to 21 days. No side effects were noted. The clinical effects appeared to be very satisfactory. There were 2 deaths; the first had received colistin only during the last 24 hours of life; the second, an infant with Roger's disease and bronchopneumonia showed a negative stool after three days of treatment. The authors point out that neomycin, which had been very effective in the prior year against *E. coli* 0111 B4, rapidly became attenuated in its effect. Polymyxin and colistin showed a distinct effect in this study.

Breton et al³ found serologically determined strains of *E. coli* occupied a prominent place in enteric infection in infancy. *E. coli* 0111 B4 was the most frequently encountered (75 per cent), strain 055 B12 occurred in only 13 per cent of cases, and strain 026 B6 in 8 per cent. Other strains were very rare (4 per cent). They found (1953–1958) the following: Gastroenteritis *E. coli* alone or in combination, 52.6 per cent; gastroenteritis from *Salmonella*, 3.9 per cent; gastroenteritis from *Shigella*, 2.6 per cent.

Origin of infections were 51.3 per cent from hospitals, 40.7 per cent outside hospitals, and 7.8 per cent unknown. Increasing resistance to streptomycin, chloramphenicol, chlortetracycline, and oxytetracycline were observed within a year of their use, and by the middle of 1955, 96 per cent of *E. coli* 0111 B4 were no longer sensitive to antibiotics and sulfonamide drugs. Coincidentally treatment became difficult, relapses occurred more frequently, and the mortality rate increased. In 1955–1956 neomycin-framycetin combination was introduced but resistance rapidly appeared. Colistin was used after 1956 and found very effective in vitro. Fifty children were treated for six to eight days in dosages of 100,000 units/Kg./day orally in two doses. The duration of therapy averaged six to eight days. There were no toxic effects. There was no increase in pathogenic staphylococci or *Candida albicans*. With *E. coli* 0111 B4, 90 per cent cure was effected in six to eight days.

Sacrez et al⁴ stated that colistin should be the first antibiotic to be used against *E. coli* 0111 B5, 0119 B14, and 055 B5.

A number of other reports in the Italian and French literature cited instances of diarrheal disturbances in infancy and childhood with beneficial effect from colistin and no evidence of toxic side effects.⁵ Ruf¹⁰ presented a comprehensive monograph with detailed discussion of colistin in diarrheal disturbances.

MATERIAL AND METHOD

This study was carried out in the infant diarrhea ward of Cook County Children's Hospital. The upper age limit of these infants in this area of the hospital is one year. This report covers two groups of infants. The first group of 103 infants was divided alternately according to admission into a test and a control series. Where admissions occur over a 24 hour period in a large hospital with varying house officers on duty, strict control is, of course, impossible, since critically ill infants will often be treated vigorously and without regard to experimental conditions, so the two groups are not strictly parallel. The second series consisted of a group of infants in whom pathogenic microorganisms had been isolated in the stool.

The drug, colistin sulfate, was administered orally immediately upon admission of the infant with a diarrheal disturbance in dosages approximating 3 to 5 mg./Kg.

TABLE I
Age Distribution

	No.	Birth to 4 weeks	1-3 mo.	3-6 mo.	6-9 mo.	9-12 mo.
Colistin sulfate	54	13	15	13	10	3
Control	49	16	12	11	6	4
Series 2 (specific diarrheas)	25	7	6	5	3	4
Total	123	36	33	29	19	11

body weight per day in three or four doses. The general management of the diarrhea was similar in all cases and consisted of withholding of oral food and water for 24 to 48 hours, administration of water and electrolyte intravenously as indicated, and in the control group, the administration of some antibiotic as indicated. Stool cultures were followed closely, and when an organism considered pathogenic was isolated, in vitro sensitivity tests by the disc method were run against colistin sulfate as well as other commonly used antibiotic and chemotherapeutic agents.

RESULTS

In series 1 there was 1 death in the "control" group. This was a Mongoloid male infant 4 weeks of age who entered with a severe diarrhea in shock and severe acidosis (carbon dioxide combining power 13 vol. per cent). He received intravenous tetracycline and chloramphenicol followed by oral neomycin, and his diarrhea slowly improved. The stools showed nonpathogenic *E. coli*, *Proteus morgagnii*, and *Pseudomonas aeruginosa*. On the eighteenth hospital day, he went into congestive heart failure and was digitalized. He died on the twenty-fifth day. Autopsy revealed an atrioventricularis communis. All other infants in this group recovered.

The age distribution of these infants is shown in table I. It will be noted that the three groups are very similar; three quarters of the infants were less than 6 months of age. These are infants of the underprivileged and are more than 90 per cent Negro.

The final series of 25 infants, all of whom yielded a pathogenic organism in their stools and all of whom were treated with colistin sulfate orally also had 1 fatality. This female infant was brought into the hospital by an aunt a few hours after birth at home. She weighed 5 pounds, 4 ounces and revealed no abnormal findings on admission. At the age of 3 days, she was transferred to a "boarder's" ward. At the age of 2 weeks, she developed a diarrhea and was sent to the infant diarrhea unit. She was not severely ill, required no intravenous medication and received tetracycline, 30 mg. four times a day orally. She was doing fairly well when on the eighteenth day of life she suddenly developed a spell of apnea and died. Autopsy revealed "prematurity and atelectasis" only. Stool culture revealed *E. coli* 055 which was sensitive in vitro to colistin sulfate and to neomycin but resistant to chloramphenicol, streptomycin, tetracycline, and sulfisoxazole. Thus, in the entire group of 128 infants, the over-all mortality was 1.58 per cent.

Table II shows the numbers of infants revealing pathogenic organisms in stool culture. In the series 1 of colistin-treated cases, these were as follows: *Salmonella*

TABLE II

Infants with Diarrhea Revealing Pathogenic Microorganisms in Their Stools

		Pathogens		
		Total	No.	%
Series 1	Colistin treated	53	18	34
	Control group	49	16	32.6
Series 2 (selected)	Colistin treated	25	25	100

derby, 1; *Shigella flexneri*, 1; hemolytic *Staphylococcus aureus* coagulase positive, 2; and serum specific *E. coli*, 17. In three instances discharge cultures returned positive, two *E. coli* 0119 and one *E. coli* 0125. In all other instances organisms cleared from the stools. Three of these infants showed organisms resistant in vitro to colistin sulfate. All of these responded to therapy clinically and showed negative cultures on discharge. In the series 1 control cases, one *Sh. flexneri*, one hemolytic *Staph. aureus* coagulase positive and 14 serum specific *E. coli* were isolated. Of these the hemolytic *Staph. aureus* was resistant in vitro to colistin sulfate and tetracycline, an *E. coli* 055 was resistant to tetracycline, and an *E. coli* 0125 was resistant to chloramphenicol and tetracycline.

Thirteen of these 16 control infants were treated from the first hospital day with an antibiotic; 3 received only symptomatic therapy. Tetracycline was used in 12 alone or with added chloramphenicol, and oral neomycin was used in 1. One infant received parenteral penicillin. In 2 cases, failure of response to therapy prompted initiation of colistin sulfate on the tenth and twenty-fourth hospital day respectively with rapid subsidence of symptoms and disappearance of the pathogenic organisms from the stool in both.

Table III shows pathogenic *E. coli* isolated and their in vitro sensitivities. It will be noted that, contrary to the experiences reported from France, the organisms found in this series were with one exception sensitive in vitro to neomycin. There were three that showed resistance to colistin sulfate: an *E. coli* 0119 and two strains of *E. coli* 0111. Furazolidone also showed a high degree of in vitro activity against the various strains. Of 14 strains tested, only one, an *E. coli* 026, showed resistance. In contrast to this finding, the commonly used antibiotics and sulfoxazole revealed relatively poor in vitro activity against these organisms. There were 12 strains resistant and four moderately resistant to chloramphenicol of 42 tested. With tetracycline there were 20 resistant and one moderately resistant to streptomycin. Eight of 21 strains tested were sensitive to sulfoxazole.

DISCUSSION

These observations on the oral use of colistin sulfate in diarrheal disturbances of infancy indicate that another antibiotic active against organisms commonly involved is available. This material is readily accepted and well tolerated by the young infant, and no deleterious side effects were observed. From a clinical standpoint the impression was gained that improvement was rapid, but in comparison with the

"control" group no definite advantage could be demonstrated, since these infants also responded well. There were several instances, however, in which infants were responding poorly to other broad-spectrum antibiotics and rapidly improved when oral colistin sulfate was substituted. There were, also, several instances in which parenteral administration of sodium colistinmethanesulfonate was employed in severe diarrhea and in *E. coli* septicemia with beneficial results, but since these were isolated observations they are not included in the series reported.

Bacteriological observations revealed a considerable percentage of these diarrheas to have been due to pathogenic *E. coli*. Most of these showed in vitro sensitivity to

TABLE III
Sensitivity Reactions of Pathogenic Strains Isolated from Stools

Organism	Col- istin	Neo- mycin	Tetra- cycline	Chlor- amphenicol	Strep- tomyacin	Fura- zolidone	Sulfisox- azole
<i>E. coli</i> 055	S*	S	R	S	R	S	
<i>E. coli</i> 055	S	S	S	S		S	
<i>E. coli</i> 055	S	S	R	MR	R	S	
<i>E. coli</i> 0126	S	S	S	S	S	S	
<i>E. coli</i> 055	S	S	R	MR	R	S	
<i>Salmonella derby</i>	S	S	S	S	S	S	
<i>E. coli</i> 0125	S	R	R	R	R		R
<i>E. coli</i> 0127	S	S	R	R	R	S	
<i>E. coli</i> 0125	S	S	S	S	R	S	
<i>E. coli</i> 055	S	S	S	S	S	S	
<i>E. coli</i> 026	S		S	S		S	
<i>E. coli</i> 0119	S	S	S	S	S	S	
<i>E. coli</i> 0119	S		S	S	R	S	
<i>E. coli</i> 0119	S	S		S	S		S
Hemolytic <i>Staphylococcus</i> coagulase positive	R		R	S			
<i>E. coli</i> 0125	S	S	MR	S			
<i>E. coli</i> 055	S	S	S	S	S	S	
<i>E. coli</i> 055	S	S	R	S	R	S	
<i>E. coli</i> 0111	S	S	S	S	S	S	
<i>E. coli</i> 0125	S	S	S	S	S		
<i>E. coli</i> 055	S		R	R	R	S	
<i>E. coli</i> 0119	S		R	S	S	S	
<i>E. coli</i> 0119	S		S	S	R	S	
<i>E. coli</i> 055	S		S	S	R	S	
<i>E. coli</i> 026	S	S	R	R	R	S	S
<i>E. coli</i> 026	S	S	S	S	R	R	
<i>E. coli</i> 0119	S	S	R	MR	MR		S
<i>E. coli</i> 055	S	S	R	R	R		R
<i>E. coli</i> 0119	S	S	R	S	R		R
<i>E. coli</i> 0111	S	S	S	S	R		S
<i>E. coli</i> 0111	S	S	R	S	R		S
<i>E. coli</i> 0119	S	S	S	S	S		S
<i>E. coli</i> 0111	S	S	S	R	R		R
<i>E. coli</i> 0111	S		R	R	R		R
<i>E. coli</i> 0111	S	S	S	R	R		R
<i>E. coli</i> 055	S	S	R	S	S		S
<i>E. coli</i> 0111	R	S	S	S	R		R
<i>E. coli</i> 055	S	S	R	R	R		R
<i>E. coli</i> 055	S	S	R	R	R		S
<i>E. coli</i> 0111	R	S	R	R	R		R
<i>E. coli</i> 055	S	S	S	R	R		R
<i>E. coli</i> 0119	R	S	R	MR	R		S
<i>E. coli</i> 0111							

* S, sensitive; R, resistant; MR, moderately resistant.

colistin, but the few that revealed resistance seemed to respond clinically as well as the remainder. In most instances, pathogenic organisms were eradicated from the stool: in the 2 instances in which discharge cultures were positive, further therapy readily converted them to negative. Contrary to the experiences reported in France, in vitro sensitivity of specific *E. coli* to neomycin was very high in this series, as it was to furazolidone. The commonly employed broad-spectrum antibiotics, tetracycline, chloramphenicol, streptomycin, as well as sulfisoxazole, revealed many strains of serum specific *Escherichia* resistant in in vitro tests. It would appear that emergence of resistant strains is a reflection of wide usage of drugs in general practice and that neomycin and colistin may well demonstrate similarly increasing loss of effectiveness should wide usage develop. For this reason so-called prophylactic use of these drugs in institutional environments on an empiric basis is undesirable and should not be employed. Where a pathogenic organism is isolated and is of a type probably sensitive to the drug, as an *E. coli* for example, it should, of course, be vigorously employed. We are quite sure several of our cases were acquired in the hospital, particularly in the "boarder's" ward, and constant vigilance with frequent stool cultures to detect carriers in such areas is necessary.

SUMMARY AND CONCLUSION

One hundred and twenty-eight infants less than 1 year of age with acute diarrhea were studied. Seventy-eight of these were treated with oral colistin sulfate; the remainder received a conventional broad-spectrum antibiotic either orally or by intravenous or intramuscular route. The over-all mortality was 1.58 per cent.

Pathogenic microorganisms were isolated in about one third of the infants observed. These revealed in vitro sensitivity to colistin sulfate in all but 3 instances of 48 organisms tested (6.9 per cent). There was also high in vitro sensitivity to neomycin and furazolidone, but many of the strains were resistant to tetracycline, chloramphenicol, streptomycin, and sulfisoxazole.

Colistin sulfate appears to be a safe and effective antibiotic for the therapy of acute diarrhea in infancy. It may also be used to eradicate pathogenic *E. coli* from the stools of carriers.

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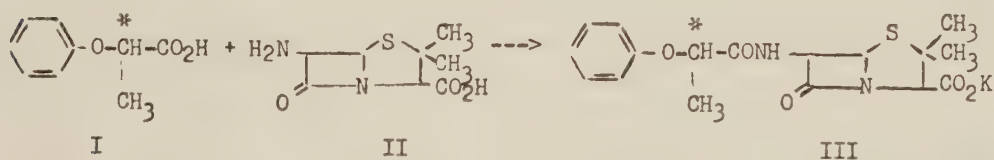
Synthesis of Potassium Penicillin-152 [Potassium (α -Phenoxyethyl)-Penicillin] and Both Diastereoisomers

Y. G. PERRON, L. B. CRAST, W. J. GOTTSTEIN, W. F. MINOR,
AND L. C. CHENEY

Research Division, Bristol Laboratories Inc., Syracuse, N. Y.

The recent disclosure by Batchelor et al¹ that 6-aminopenicillanic acid could be obtained by fermentation prompted us to synthesize a variety of novel penicillins not amenable to production by biosynthetic methods.² This paper deals with the synthesis of a new penicillin, potassium α -phenoxyethyl penicillin,* which possesses valuable therapeutic properties.

This new penicillin (see formula III) is prepared by N-acylation of 6-aminopenicillanic acid (II) with DL- α -phenoxypropionic acid (I); the reaction product is isolated as the potassium salt.



Because of the presence of the asymmetric carbon (marked by an asterisk in the formula) in the side chain of the molecule, compound III is a mixture of two diastereoisomeric forms. Racemic α -phenoxypropionic acid has been resolved^{3,4} and the two optical isomers thus obtained have been used to synthesize each of the diastereoisomeric forms of this new penicillin. The distinctive physical properties of these two diastereoisomers are described in the experimental section of this paper. The infrared absorption spectra of the two crystalline diastereoisomers are shown in figure 1. The two spectra show an almost analogous absorption in the functional group region of the spectrum while the location and intensity of the bands in the "finger print" region are quite distinctive. Both isomeric α -phenoxyethyl penicillins show marked antimicrobial properties, which are described by Gourevitch et al.⁵ The absolute configurations of the two diastereoisomers have not been definitely established.⁴

EXPERIMENTAL RESULTS

Preparation of Potassium DL- α -Phenoxyethyl Penicillin. DL- α -PHENOXYPROPIONYL CHLORIDE. In a 2 liter three-necked flask equipped with a stirrer, condenser (calcium chloride tube), and a dropping funnel, 166 Gm. of DL- α -phenoxypropionic acid, 800 ml. of benzene, and 2 ml. of pyridine were added. The mixture was stirred and heated to reflux. Then, 107.4 ml. of thionyl chloride was added dropwise during 30 minutes. When the addition was completed, refluxing was con-

* The trade name of Bristol Laboratories Inc. for potassium α -phenoxyethyl penicillin is Syncillin.

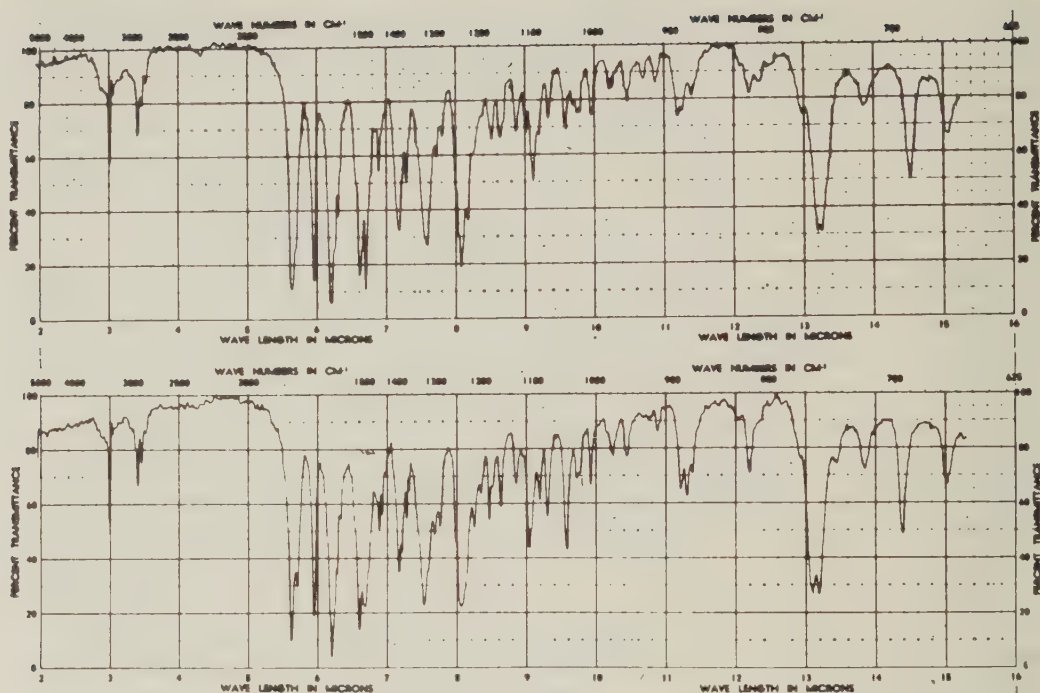


FIG. 1. Upper curve: potassium D- α -phenoxyethyl penicillin; lower curve: potassium L- α -phenoxyethyl penicillin.

tinued for one hour. The benzene was taken off under reduced pressure and the oily residue was distilled in vacuo yielding 146 Gm. of material; b.p. 114-115 C. at 18 mm.

Potassium DL- α -PHENOXYETHYL PENICILLIN. To a cooled and stirred solution of 54 Gm. (0.25 mole) of 6-aminopenicillanic acid in 1.2 liter of water containing 105 Gm. (1.25 mole) of sodium bicarbonate, a solution of 60 Gm. (0.325 mole) of DL- α -phenoxypropionyl chloride in 100 ml. of acetone was added in one minute. The resulting mixture was stirred vigorously during 20 minutes while the temperature was kept at 10 to 15 C. The resulting clear solution was extracted twice with 300 ml. portions of methyl isobutyl ketone, the organic extracts being discarded. The clear aqueous solution was covered with 500 ml. of methyl isobutyl ketone, cooled to 5 to 10 C., and acidified to pH 2 with a cold 5 M sulfuric acid solution. The methyl isobutyl ketone extract was separated, washed with cold water, and dried for 10 minutes over anhydrous sodium sulfate. After filtration, 100 ml. of a 50 per cent solution of potassium 2-ethylhexanoate in *n*-butanol was added. The white crystalline material, which separated almost immediately, was collected by filtration, washed on the filter with a little dry acetone and dried. There was obtained 76 Gm. (76 per cent) of colorless crystals which decomposed at 230 to 231 C.

Analytical. Calculated for $C_{17}H_{19}N_2O_5SK$: C, 50.75; H, 4.78; N, 6.98. Found: C, 50.81; H, 4.88; N, 7.15.

RESOLUTION OF DL- α -PHENOXYPROPIONIC ACID. (+)- α -PHENOXYPROPIONIC ACID. The method of Fourneau and Sandulesco³ was adopted to prepare this optical isomer. Starting with 8 oz. of yohimbine hydrochloride, 12 Gm. of optically pure

(+)- α -phenoxypropionic acid was obtained; m.p., 86.5 to 88 C., $[\alpha]_D^{25} = +39.8^\circ$ ($c = 1$ per cent in absolute alcohol) (literature³ $[\alpha]_D^{20} = +39.3^\circ$).

(-)- α -PHENOXYPROPIONIC ACID. This isomer was isolated by a procedure essentially that of Fredga and Matell.⁴ A product was obtained by evaporation under reduced pressure of the mother liquor from the isolation of the (+)- α -phenoxypropionic acid yohimbine salt. This was treated with dilute sulfuric acid and extracted with ether to give an acid having $[\alpha]_D^{24} = -25.3^\circ$ ($c = 1$ per cent in absolute alcohol). When 27 Gm. of this material was slurried with chloroform at room temperature and filtered there was obtained 10 Gm. of insoluble DL- α -phenoxypropionic acid (m.p. 117 to 119 C.), and 17 Gm. of soluble material by evaporation of the chloroform. The latter was slurried with 150 ml. of boiling cyclohexane and filtered, removing 1.4 Gm. of insoluble material. Evaporation of the filtrate left 14.7 Gm. of (-)- α -phenoxypropionic acid; m.p. 86 to 87 C., $[\alpha]_D^{23.5} = -39.5^\circ$ ($c = 1$ per cent in absolute alcohol) (literature⁴ $[\alpha]_D^{20} = -39.3^\circ$).

POTASSIUM D- α -PHENOXYETHYL PENICILLIN. A solution was prepared by mixing 8.3 Gm. (0.05 mole) of (+)- α -phenoxypropionic acid, 40 ml. of dry *p*-dioxane, 20 ml. of dry acetone, and 8 ml. of triethylamine. To this stirred and cooled solution (about 0 C.) was added dropwise, during 10 to 15 minutes, 6.8 Gm. (0.05 mole) of isobutyl chloroformate in 10 ml. of *p*-dioxane while the temperature was maintained below 10 C. After the addition was completed the mixture was stirred and cooled during 10 minutes after which time a solution of 10.8 Gm. (0.05 mole) of 6-aminopenicillanic acid in 50 ml. of water and 8 ml. of triethylamine was added rapidly. The resulting solution was stirred 15 minutes at about 10 C. and then two hours at room temperature. After dilution with an equal volume of water the reaction mixture was extracted twice with 100 ml. portions of ether, the ethereal extracts being discarded. The clear aqueous solution was covered with 150 ml. of ether, cooled to 10 C., and acidified to pH 2 with a cold 5 *M* sulfuric acid solution. The ethereal solution was separated, washed with cold water and dried for 10 minutes over anhydrous sodium sulfate. After filtration 25 ml. of a 50 per cent solution of potassium 2-ethylhexanoate in *n*-butanol was added. The white crystalline material which separated was collected by filtration and recrystallized once from 10 per cent aqueous *n*-butanol and once again from 10 per cent aqueous acetone. This procedure afforded 5 Gm. of pure potassium D- α -phenoxyethyl penicillin which decomposed at 230 to 231 C., $[\alpha]_D^{24} = +251^\circ$ ($c = 1$ per cent in water).

Analytical. Calculated for $C_{17}H_{19}N_2O_5SK$: C, 50.75; H, 4.78; N, 6.98. Found: C, 50.62; H, 4.97; N, 7.05.

POTASSIUM (L- α -PHENOXYETHYL) PENICILLIN. The preparation of this material is carried out exactly as described previously for the isomeric potassium D- α -phenoxyethyl penicillin. The yield of pure material after one crystallization from 20 per cent aqueous *n*-butanol is 9.5 Gm.; decomposition 238 to 239 C., $[\alpha]_D^{24} = +218^\circ$ ($c = 1$ per cent in water).

Analytical. Calculated for $C_{17}H_{19}N_2O_5SK$: C, 50.75; H, 4.78; N, 6.98. Found: C, 50.92; H, 5.15; N, 6.83.

SUMMARY

A new penicillin, namely, potassium α -phenoxyethyl penicillin, has been prepared

by the condensation of α -phenoxypropionic acid with 6-aminopenicillanic acid and isolated as the potassium salt. The two possible disastereoisomers of this new penicillin have also been prepared and their distinctive physical properties are described.

ACKNOWLEDGMENTS

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Microbiological Studies on Potassium Penicillin-152 [Potassium (α -Phenoxyethyl)-Penicillin]

A. GOUREVITCH, G. A. HUNT, AND J. LEIN

Research Division, Bristol Laboratories Inc., Syracuse, N. Y.

A new penicillin, potassium α -phenoxyethyl penicillin,* has been prepared by reaction of α -phenoxypropionyl chloride with 6-aminopenicillanic acid.³ Attempts to produce it by fermentation using a suitable precursor have been unsuccessful. When a racemic mixture of acid chlorides is used in the chemical synthesis, a mixture of D- α -phenoxyethyl penicillin and of L- α -phenoxyethyl penicillin is obtained. Pure D- α -phenoxyethyl penicillin and L- α -phenoxyethyl penicillin have also been prepared from optically pure dextro and levo α -phenoxypropionic acids, respectively. In the following text, α -phenoxyethyl penicillin will refer to the mixture of the two diastereoisomers, whereas the pure isomers will be referred to as D- or L- α -phenoxyethyl penicillin, respectively. Microbiological comparisons of these materials were made with phenoxymethyl penicillin (penicillin V) and benzylpenicillin (penicillin G). All materials were used as potassium salts.

ANTIBACTERIAL SPECTRUM

The minimum inhibitory concentration was determined for a variety of microorganisms using serial dilution techniques. The inoculum was a 10^4 dilution of an overnight broth culture except for those cultures which grew slowly, in which case a 10^3 dilution was used. The presence or absence of growth was determined after 18 hours at 37 C. The results are listed in table I.

It will be seen from the data that the D- α -phenoxyethyl penicillin under these conditions is somewhat less active than the L- α -phenoxyethyl penicillin. Furthermore, the mixture, α -phenoxyethyl penicillin, which represents production material, is at least as active as the L-isomer and appears to be more active in some cases. The activity of α -phenoxyethyl penicillin is similar to that of penicillin V against a large variety of organisms.

EFFECT ON RESISTANT STAPHYLOCOCCAL STRAINS

When minimum inhibitory concentrations are determined against a variety of clinically isolated resistant staphylococcal strains, the inhibitory end points are invariably lower than those obtained with penicillin G or V. This is shown in table II.

While the minimum inhibitory concentrations with α -phenoxyethyl penicillin appear to be considerably lower than that obtained with penicillin G or V, it is not known if the enhanced sensitivity is marked enough to be therapeutically significant.

* The trade name of Bristol Laboratories Inc. for potassium α -phenoxyethyl penicillin is Syncillin.

TABLE I

Comparison of Minimum Inhibitory Concentrations of Penicillins

Organism	Medium, heart infusion broth	Minimum inhibitory concentration				
		D- α -Phen- oxyethyl penicillin	L- α -Phen- oxyethyl penicillin	α -Phen- oxyethyl penicillin	Penicillin V	Penicillin G
<i>Bacillus anthracis</i> *		0.25	0.06	0.03	0.03	0.15
<i>Bacillus cereus</i> *		100	12.5	25	25	6.25
<i>Bacillus circulans</i> ATCC 9961		6.25	6.25	6.25	3.1	1.6
<i>Corynebacterium xerosis</i> *		0.125	0.06	0.03	0.03	0.015
<i>Diplococcus pneumoniae</i> † + 10% serum		0.06	0.06	0.06	0.03	0.03
<i>Escherichia coli</i> ATCC 8739		>100	>100	>100	>100	50
<i>Gaffkya tetragena</i> *		0.03	0.015	0.015	0.007	0.015
<i>Micrococcus flavus</i>		0.125	0.015	0.015	0.007	0.007
<i>Salmonella paratyphi</i> A‡		50	25	25	12.5	0.4
<i>Salmonella typhosa</i> *		>100	>100	>100	>100	50
<i>Sarcina lutea</i> ATCC 10054		0.12	0.007	0.007	0.007	0.0035
<i>Shigella sonnei</i> *		100	100	100	25	12.5
<i>Staphylococcus aureus</i> 209P		0.125	0.06	0.03	0.03	0.03
<i>Staphylococcus aureus</i> var. Smith		0.125	0.03	0.03	0.03	0.03
<i>Streptococcus agalactiae</i> ATCC 1077		0.06	0.03	0.03	0.03	0.015
<i>Streptococcus dysgalactiae</i> ATCC 9926		0.06	0.03	0.03	0.03	0.015
<i>Streptococcus faecalis</i> PCI 1305		25	6.25	6.25	25	1.6
<i>Streptococcus pyogenes</i> C203 + 10% serum		0.06	0.06	0.06	0.015	0.015
<i>Streptococcus pyogenes</i> Digonnet F§ + 10% serum		0.15	0.03	0.06	0.03	0.03
<i>Streptococcus pyogenes</i> #2320 + 10% serum		0.06	0.06	0.03	0.03	0.007
<i>Streptococcus pyogenes</i> #23586 + 10% serum		0.06	0.06	0.06	0.03	0.015
<i>Vibrio comma</i> *		25	50	25	12.5	1.6

* Cultures from Yale collection brought to Bristol Laboratories by Dr. George Valley.

† Type II, St. Louis City Hospital, D-11008. Brought to Bristol Laboratories by Dr. G. A. Hunt.

‡ Department of Health, Hartford, Conn.

§ Strain received from Pasteur Institute, Paris, France.

|| Clinical strains from Memorial Hospital, Syracuse, N. Y.

TABLE II

Comparison of Minimum Inhibitory Concentrations of Penicillins Using *Staph. aureus* Penicillin Resistant Strains of Clinical Origin

<i>Staph. aureus</i> strain no.	Minimum inhibitory concentration, $\mu\text{g./ml.}$				
	D- α -Phenoxy- ethyl penicillin	L- α -Phenoxy- ethyl penicillin	α -Phenoxy- ethyl penicillin	Penicillin V	Penicillin G
52-34	3.1	1.6	0.8	6.2	12.5
52-75	6.2	3.1	3.1	25	50
WR188	3.1	3.1	1.6	12.5	12.5
BRL J	0.8	0.8	0.8	1.6	3.1
BRL O	1.6	0.8	0.8	12.5	25

TABLE III

Effect of Bacillus cereus Penicillinase on Different Penicillins

Penicillin	Inactivation, %				
	0 min.	15 min.	30 min.	60 min.	120 min.
α -Phenoxyethyl penicillin	0	40	56	88	100
Penicillin V	0	100	100	100	100
Penicillin G	0	81	100	100	100

EFFECT OF PENICILLINASE

The rates of inactivation of α -phenoxyethyl penicillin, penicillin G, and penicillin V by *Bacillus cereus* penicillinase were determined. The results (table III) gave the percentage inactivation by penicillinase A at 37 C. It will be seen that α -phenoxyethyl penicillin is considerably more resistant to *B. cereus* penicillinase than penicillin V or G.

Similar results were obtained using staphylococcal penicillinase. The latter was obtained by growing penicillin resistant *Staphylococcus aureus* strain 52-75 in the presence of penicillin G in Trypticase soy broth and filtering off the cells. The filtered broth served as the source of penicillinase.

The results of inactivation of the penicillins by staphylococcal penicillinase are given in table IV.

EFFECT OF SERUM

The minimum inhibitory concentrations of the penicillins against the Smith strain of *Staph. aureus* were determined in heart infusion broth and in 100 per cent serum in two independent experiments. The data are given in table V. It will be noticed that in the presence of serum, D- α -phenoxyethyl penicillin is as active as L- α -phenoxyethyl penicillin in contrast to heart infusion broth.

ACID STABILITY STUDIES

The stability of α -phenoxyethyl penicillin was compared with penicillin V and G at three temperatures. The penicillins were dissolved in 0.002 M citrate buffer

TABLE IV

Effect of Staphylococcal Penicillinase on Different Penicillins

	Inactivation in 1 hr., %
α -Phenoxyethyl penicillin	64
D- α -Phenoxyethyl penicillin	73
L- α -Phenoxyethyl penicillin	71
Penicillin V	100
Penicillin G	100

TABLE V

*Effect of Serum on Minimum Inhibitory Concentration of Penicillins
Using Staphylococcus aureus (Smith)*

Penicillin	Minimum inhibitory concentration, $\mu\text{g./ml.}$			
	Experiment 1		Experiment 2	
	Heart infusion broth	100% serum	Heart infusion broth	100% serum
D- α -Phenoxyethyl penicillin	0.25	0.25	0.25	0.25
L- α -Phenoxyethyl penicillin	0.03	0.25	0.03	0.25
α -Phenoxyethyl penicillin	0.03	0.5	0.06	0.25
Penicillin V	0.03	0.125	0.03	0.5
Penicillin G	0.02	0.125	0.03	1.0

at pH 2 and 3 and the percentage decrease in activity of samples kept at 5, 25, and 37 C. determined by plate assays. The values obtained are listed in table VI.

The data indicate that α -phenoxyethyl penicillin and penicillin V have essentially equivalent acid stability, both being considerably more stable than penicillin G.

EFFECT OF MEDIUM AND PH

The minimum inhibitory concentration using *Staph. aureus* was determined in three different media, which were adjusted to different pH values. The media used were heart infusion broth, nutrient broth, and nutrient broth supplemented with 0.5 per cent yeast extract (table VII). It will be seen that there is only a slight

TABLE VI

Effect of Low pH and Different Temperatures on Penicillins

Temp., C.	Hours	Decrease in activity, %					
		α -Phenoxyethyl penicillin		Penicillin V		Penicillin G	
		pH 2	pH 3	pH 2	pH 3	pH 2	pH 3
5	0	0	0	0	0	0	0
	0.5	8	5	8	—	6	0
	1	—	8	4	5	19	6
	5	3	3	7	7	76	3
25	0	0	0	0	0	0	0
	0.5	8	10	0	17	77	9
	1	18	13	30	20	93	31
	5	36	17	30	—	100	75
37	0	0	0	0	0	0	0
	0.5	15	11	17	21	97	27
	1	35	13	21	18	100	—
	5	75	26	72	15	100	94

effect of medium ingredients and pH and that α -phenoxyethyl penicillin and penicillin V behave alike in this respect.

DEVELOPMENT OF RESISTANCE

In order to estimate how readily resistance may develop to α -phenoxyethyl penicillin, an experiment was done to determine the number of resistant cells in a large population of normal *Staph. aureus* strain 209P. Platings of 10^8 cells were done on Petri plates containing increasing concentrations of the penicillins. The number of colonies growing up is indicative of the number of cells resistant at least to that level of penicillin (table VIII). The number of resistant cells decrease rapidly as the concentration increases and there is no evidence of "tailing," i.e., a few colonies growing on plates containing a high concentration of antibiotic, such as is obtained

TABLE VII
Effect of Medium and pH on Minimum Inhibitory Concentration

Penicillin	Minimum inhibitory concentration, $\mu\text{g.}/\text{ml.}$											
	Heart infusion broth				Nutrient broth				Nutrient broth 0.5% yeast extract			
	pH6.5	pH7.0	pH7.5	pH8.0	pH6.5	pH7.0	pH7.5	pH8.0	pH6.5	pH7.0	pH7.5	pH8.0
α -Phenoxyethyl penicillin	0.03	0.03	0.06	0.06	0.03	0.06	0.06	0.06	0.007	0.03	0.03	0.06
Penicillin V	0.03	0.06	0.06	0.13	0.03	0.03	0.06	0.06	0.03	0.03	0.06	0.06

TABLE VIII
Staphylococcus aureus Survivors Obtained on Plates of Heart Infusion Agar Containing Increasing Concentrations of Penicillins

Penicillin	Number of colonies* at penicillin concentration on plates ($\mu\text{g.}/\text{ml.}$)				
	0.0078	0.0156	0.031	0.062	0.12
α -Phenoxyethyl penicillin	>1000	>1000	>300	1	0
Penicillin V	>1000	100	0	0	0
Penicillin G	>1000	>300	20	0	0

*Each plate received 10^8 cells.

TABLE IX
Comparison of CD_{50} Values of Individual Diastereoisomers and α -Phenoxyethyl Penicillin Using Staphylococcus aureus

Penicillin	CD_{50} , mg./Kg.			
	No. 1	No. 2	No. 3	Geometric mean
D- α -Phenoxyethyl penicillin	0.85	0.72	1.25	0.92
L- α -Phenoxyethyl penicillin	0.35	0.60	0.70	0.53
α -Phenoxyethyl penicillin	0.18	0.40	0.45	0.32

TABLE X

Comparison of CD₅₀ Values of Different Penicillins Using Staphylococcus aureus

Penicillins	CD ₅₀ mg./Kg.							Geometric mean
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	
α -Phenoxyethyl penicillin	0.46	0.62	0.18	0.45	0.45	0.4	0.4	0.41
Penicillin V	0.62	1.90	0.6	0.26	0.7	1.2	0.85	0.75
Penicillin G	1.0	1.9	0.64	1.1	0.9	1.2	0.9	1.04

with streptomycin. Evidently resistance development will be expected to occur at about the same rate for all three penicillins.

IN VIVO MOUSE PROTECTION TESTS

Comparative animal protection tests were carried out using *Staph. aureus* Smith as the infecting organism. The infective dose of organism used was 100-fold the number necessary to kill half the animals (LD₅₀). Mucin was used to potentiate the infection. Details of technique have been presented previously.² The antibiotics were given intramuscularly at the time of infection and the amount of antibiotic necessary to cure half the animals was determined (CD₅₀). Ten animals were used in each dosage group. The data from three independent experiments are listed in table IX.

It is of considerable interest to note that the activity of the mixture, namely, α -phenoxyethyl penicillin, is greater than that of even the pure L- α -phenoxyethyl penicillin. While this relationship is consistently observed, the difference was not great enough to be significant statistically at the 0.05 probability level. The fact that the lowest CD₅₀ values are obtained with the α -phenoxyethyl penicillin does, however, indicate that it is the preferred therapeutic material.

CD₅₀ comparisons of the α -phenoxyethyl penicillin were made with penicillin G and penicillin V using *Staph. aureus* as the infecting organism. Data are given in table X.

It will be noted that under the conditions of this in vivo test the α -phenoxyethyl penicillin was more effective than penicillin V or G. This difference is statistically significant both at the 0.05 and 0.01 probability level.

Animal protection tests were also carried out using *Diplococcus pneumoniae* and *Streptococcus pyogenes* (Digonnet F) as the infecting organisms. There is greater variability in the CD₅₀ determinations carried out with these organisms. The CD₅₀ from several experiments and the geometric average are presented in table XI. Because of the variability of these CD₅₀ tests, differences between α -phenoxyethyl penicillin and penicillin V were not thought to be significant. This was borne out by statistical analysis of the data.

An attempt was made to determine if α -phenoxyethyl penicillin would cure an infection caused by the 52-75 strain of resistant *Staph. aureus*. No cures were obtained at doses of 500 mg./Kg.

TABLE XI

Comparison of CD_{50} Values of Penicillins Using *Diplococcus pneumoniae* and *Streptococcus pyogenes* (Digonnet F)

Infection	Penicillin	CD_{50} , mg./Kg.						Geometric mean
		I	II	III	IV	V	VI	
<i>D. pneumoniae</i>	α -Phenoxyethyl penicillin	1.8	15.0	3.2	4.8	28.0	6.0	6.4
	Penicillin V	0.9	1.2	4.2	0.9	5.2	24.0	2.8
	Penicillin G	—	4.5	32.0	6.5	19.0	11.0	11.4
<i>Str. pyogenes</i> (Digonnet F)	α -Phenoxyethyl penicillin	5.0	2.4	5.0	5.4	3.4	9.0	4.6
	Penicillin V	4.0	1.9	16.0	2.0	5.2	7.0	4.6
	Penicillin G	10.0	2.3	12.5	6.4	13.0	7.2	7.5

DISCUSSION

Both the in vitro and in vivo results indicate that the diastereoisomeric mixture is more active than even the most active component. There must evidently be some interaction of the two components with the microorganism resulting in this greater activity. A similar observation was reported for the two isomers of cycloserine. The natural isomer is configuratively related to D-serine but it has also been found that the L-isomer is microbiologically active. Smrt et al⁴ and Trivellato⁵ reported that the racemic mixture was more active in vitro than the most active isomer. Ciak and Hahn¹ also reported similar findings and presented evidence showing that the mode of action of each isomer was different. A more detailed analysis of the inhibition by each of the diastereoisomers of the new penicillin will have to be made before the reason for the observed effects will become apparent.

SUMMARY

The microbiological properties of a new penicillin, potassium α -phenoxyethyl penicillin, and its individual diastereoisomers were studied. It was found that the diastereoisomeric mixture is more active than the most active component by in vitro and in vivo tests.

The new penicillin is acid stable and has a spectrum similar to penicillin V. It inhibits resistant *Staphylococcus* at lower levels than penicillin G or V and is less readily inactivated by *B. cereus* and staphylococcal penicillinase. In in vivo infections by the intramuscular route, it appears as active as penicillin V against *D. pneumoniae* and *Str. pyogenes* infections and more active than penicillin V against *Staph. aureus* infections. It did not cure mice infected with the resistant 52-75 strain of *Staph. aureus*.

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Pharmacological Studies on Potassium Penicillin-152 [Potassium (α -Phenoxyethyl)-Penicillin]

M. H. PINDELL, D. E. TISCH, J. B. HOEKSTRA, AND J. C. REIFFENSTEIN

Research Division, Bristol Laboratories Inc., Syracuse, New York

Until recently penicillin compounds could be produced only through biological fermentation procedures, necessarily limiting the type and number of compounds that could be prepared. With the isolation and identification of 6-aminopenicillanic acid from fermentation beers by Batchelor et al,¹ it became possible to synthesize many new and novel penicillin derivatives. One of these, potassium α -phenoxyethyl penicillin* (potassium penicillin-152) (fig. 1), was prepared by Perron et al² and found to produce unusually high serum levels in human subjects following oral administration.

Because the use of this compound appears warranted in the treatment of infectious disease, pharmacological and toxicological studies have been carried out in laboratory animals.

METHODS AND RESULTS

Acute Toxicity. Acute LD₅₀ determinations on potassium α -phenoxyethyl penicillin were made in mice by intravenous, intraperitoneal, and oral routes of administration. Aqueous solutions of potassium α -phenoxyethyl penicillin and potassium penicillin G were employed for these studies. Intravenous administration was accomplished via the tail vein using 2 per cent solutions given at a rate of 0.1 ml./sec. Oral intubation was accomplished through the use of a blunted steel needle. Table I summarizes the acute mouse toxicity information.

Almost identical LD₅₀ values for potassium α -phenoxyethyl penicillin and potassium penicillin G by the intravenous route clearly demonstrate that the new compound is no more acutely toxic than penicillin G.

Toxic symptoms at near fatal or fatal doses included hyperactivity followed in some cases by "hopping" convulsions. Latent deaths seldom occurred.

Intravenous administration of 103 mg./Kg. of potassium α -phenoxyethyl penicillin (one third of the mouse intravenous LD₅₀) to unanesthetized dogs caused no apparent reaction, either immediately or subsequently.

Chronic Toxicity. RATS. Growth curves of rats dosed with potassium α -phenoxyethyl penicillin at 50 and 200 mg./Kg./day for 12 weeks were similar to that obtained with control animals given water (fig. 2). Autopsy of a portion of the animals that were sacrificed after six weeks of dosing revealed no gross or microscopic pathology in any animal.

DOGS. Prolonged daily oral administration of potassium α -phenoxyethyl penicillin to 6 dogs at 200 mg./Kg. and to 2 animals at 500 mg./Kg. resulted in no toxic manifestations. Half of these animals were sacrificed after six weeks of dosing and half received the compound for 12 weeks. At autopsy no gross or microscopic

* The trade name of Bristol Laboratories Inc. for potassium α -phenoxyethyl penicillin is Syncillin.

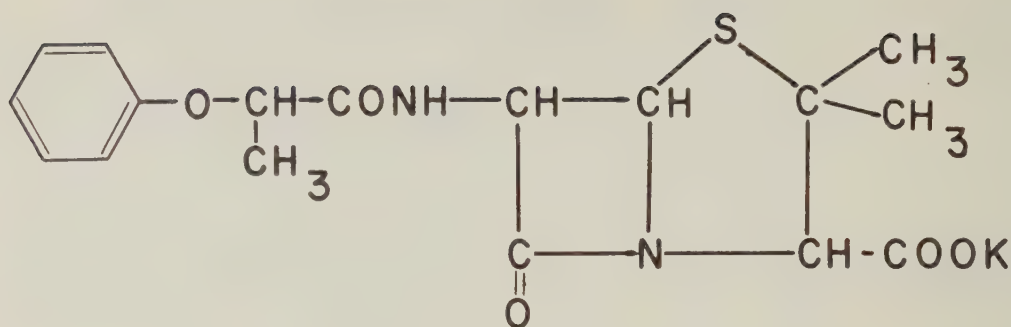


FIG. 1. Structure of potassium penicillin-152.

pathology was observed and the animals all maintained appetite and weight throughout the test periods. Laboratory indices of physiological function (urinalysis, blood cell counts, and differentials, hemoglobin, hematocrit, blood urea nitrogen, blood glucose, Bromsulphalein, and alkaline phosphatase) remained within normal limits throughout the test period. One pregnant female dog delivered a litter of normal pups after six weeks dosing at 500 mg./Kg./day.

Irritation Studies. Although the new penicillin is intended for oral use principally, local irritation studies were carried out by several techniques. It was found that intradermal administration of 0.1 ml. of a 0.5 per cent aqueous solution in rabbits resulted in no apparent discomfort or local skin reaction over a seven day observation period.

Intramuscular injection of 30 mg./Kg. in rabbits, 50 mg./Kg. in rats, and 300 mg./Kg. in dogs caused no apparent pain, discomfort, or tissue reaction.

Introduction of small amounts of irritant materials directly into the thoracic cavity elicits a marked effusion of fluid in the 24 hour period subsequent to injection. In practice, the animal is then sacrificed and the volume of effusion fluid recorded as a quantitative measure of pleural irritation.

TABLE I
Acute Toxicity in Mice

Route of administration	Dose, mg./Kg.	Dead/dosed	LD ₅₀ , mg./Kg.
<i>Potassium α-Phenoxyethyl Penicillin</i>			
Intravenous	200	0/10	312
Intravenous	250	0/10	
Intravenous	300	2/10	
Intravenous	320	7/10	
Intraperitoneal	1600	0/5	1896
Intraperitoneal	1800	1/5	
Intraperitoneal	2000	4/5	
Oral	2000	0/5	>2000
<i>Potassium Penicillin G</i>			
Intravenous	200	0/5	310
Intravenous	250	0/5	
Intravenous	300	2/5	
Intravenous	320	3/5	

Intraleural administration of 37 mg./Kg. (3.7 per cent aqueous solution) of potassium α -phenoxyethyl penicillin into each of 5 rats resulted in no effusion response whatever indicating a complete lack of irritant potential by this test.

Rats and dogs that received large daily oral doses of the compound (50 to 500 mg./Kg.) for several weeks displayed no evidence of gastrointestinal irritation at autopsy (grossly or microscopic).

Cardiovascular Studies. Intravenous administration of large doses of potassium α -phenoxyethyl penicillin to anesthetized dogs did not affect blood pressure, autonomic reactivity, or respiration significantly. Rapid administration of 50 mg./Kg. resulted in only a 12 per cent transient decrease in mean arterial pressure and no significant alteration of autonomic reactivity (response to injected epinephrine, norepinephrine, histamine, or acetylcholine).

Oral Absorption. Abraham et al³ and McDermott et al⁴ first demonstrated that sodium penicillin G was absorbed from the stomach and small intestine of experimental animals but that little was absorbed from the colon. Anderson et al⁵ showed that oral penicillin V resulted in slightly higher serum levels in rats, dogs, and monkeys than penicillin G. In a comparative study in dogs it was found that potassium α -phenoxyethyl penicillin produced higher serum levels than potassium penicillin V when both were administered at equal doses in terms of weight. Figure 3 presents the results obtained employing 4 dogs for each serum determination. Peak blood levels were reached at one hour with both penicillins but the levels of potassium α -phenoxyethyl penicillin were almost twice that of potassium penicillin V at 30 minutes demonstrating the great rapidity of oral absorption of potassium α -phenoxyethyl penicillin.

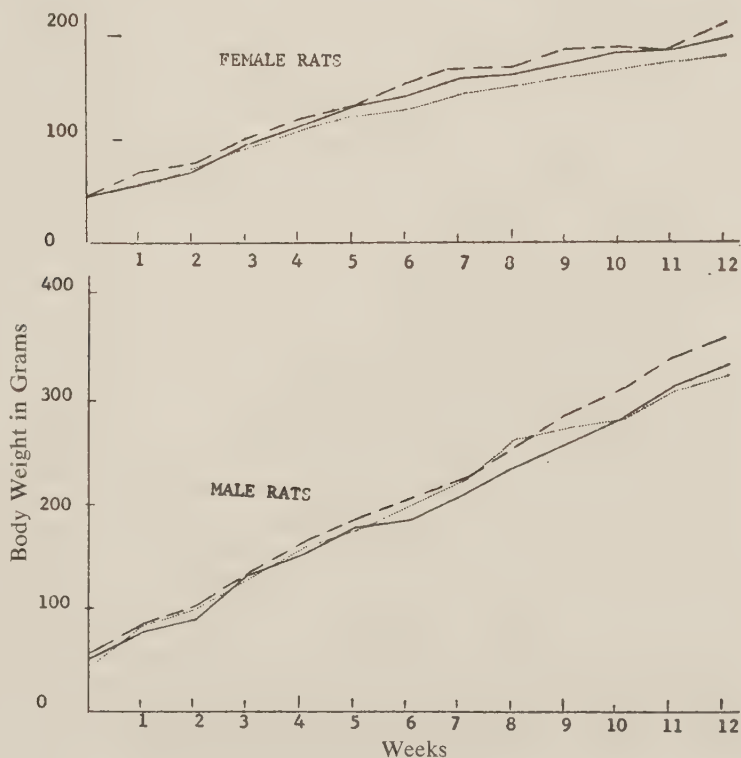


FIG. 2. Rat growth curves resulting from daily oral administration of potassium α -phenoxyethyl penicillin at 50 and 200 mg./Kg. — 50 mg./Kg./day; - - 200 mg./Kg./day; . . . control.

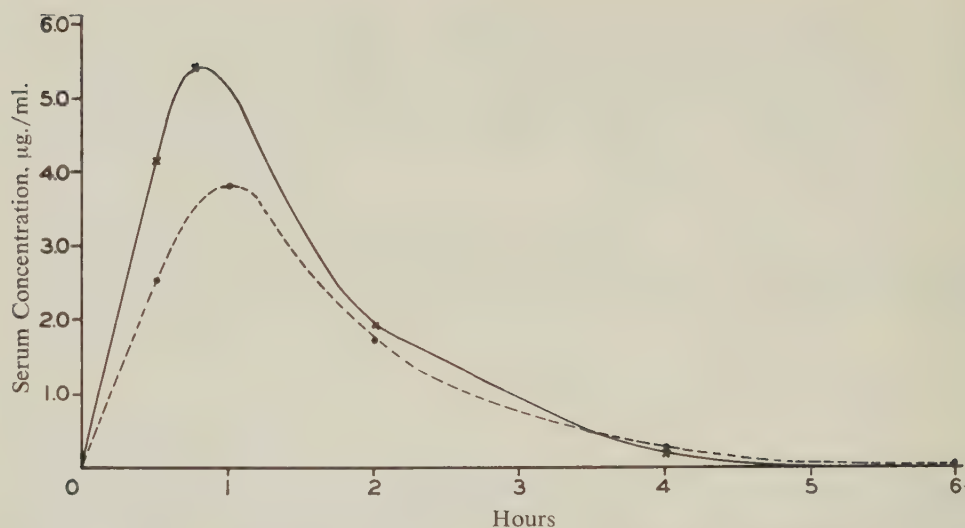


FIG. 3. Comparison of serum concentrations in dogs following oral administration of equal doses (20 mg./Kg.) of potassium α-phenoxyethyl penicillin and potassium penicillin V. — Potassium α-phenoxyethyl penicillin; — — — potassium penicillin V.

McDermott et al⁴ clearly demonstrated that oral administration of penicillin G in normal human subjects resulted in peak serum levels about one fourth to one third of those obtained following intramuscular administration of the same dose. These results demonstrated that oral absorption of penicillin G was inferior to intramuscular absorption or to put it another way, to obtain serum levels with oral penicillin G equivalent to those obtained intramuscularly, a three- to fourfold increase in oral dosage would be necessary.

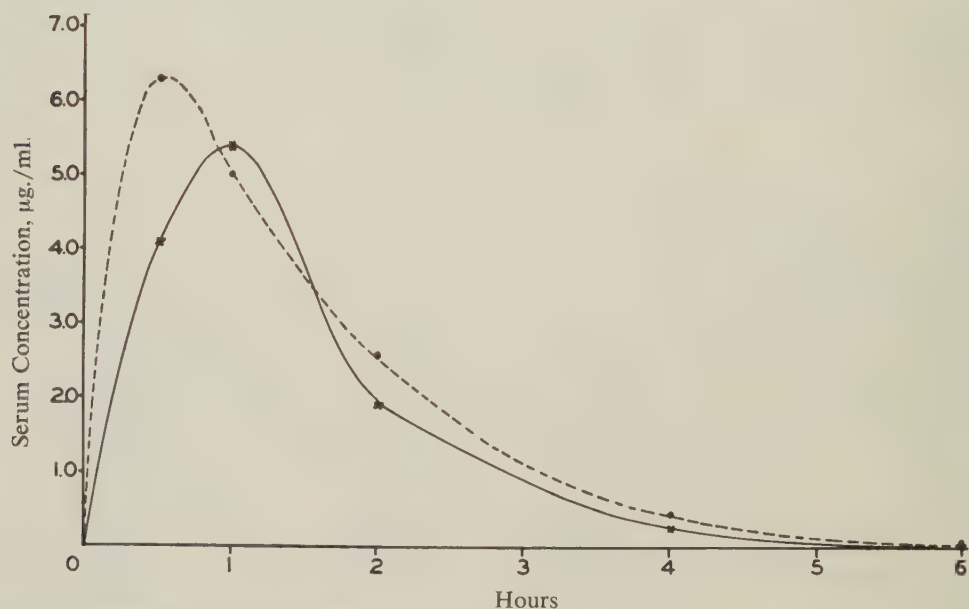


FIG. 4. Comparison of serum concentrations in dogs following intramuscular and oral administration of the same dose of potassium α-phenoxyethyl penicillin (20 mg./Kg.). — Oral administration; — — — intramuscular administration.

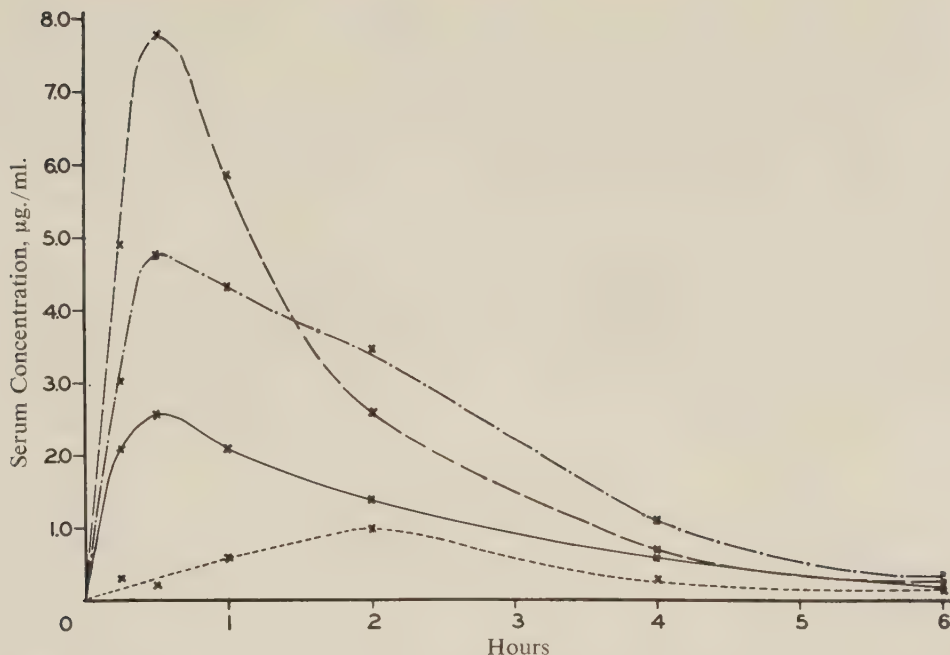


FIG. 5. Serum concentrations in dogs following administration of the same dose (20 mg./Kg.) of potassium α -phenoxyethyl penicillin at different sites along the gastrointestinal tract. —, stomach; — · —, duodenum; — — —, ileum; - - - -, colon.

Administration of equal doses (20 mg./Kg.) of potassium α -phenoxyethyl penicillin to dogs by oral and intramuscular routes resulted in the serum concentrations shown in figure 4. It is evident that although the peak serum levels were attained somewhat more rapidly following intramuscular administration than after ingestion, the serum concentrations were essentially similar by both routes.

Absorption of potassium α -phenoxyethyl penicillin from various sites along the gastrointestinal tract was studied in fasted anesthetized dogs by direct injection of 2 per cent aqueous solutions (20 mg./Kg.) into one of four sites: the stomach with the pylorus ligated, the duodenum, the ileum approximately four feet proximal to the ileocolic juncture, and the colon after enema. Serum levels of the compound were determined at 15 and 30 minutes and one, two, four, and six hours after drug administration. Figure 5 presents the results obtained. Potassium α -phenoxyethyl penicillin was absorbed from all sites tested. Absorption from the stomach, duodenum, and ileum was extremely rapid resulting in peak concentrations of 2.6, 4.8, and 7.8 $\mu\text{g./ml.}$ respectively at 30 minutes whereas absorption from the colon was slower and less efficient, a peak concentration of 1.0 $\mu\text{g./ml.}$ being attained at two hours.

Serum Binding. Dialysis was employed to determine the amount of protein binding of the new penicillin in dog serum. Direct comparisons were made with potassium penicillins G and V.

Five ml. of dog serum containing 5 mg. of the penicillin to be tested were placed in cellophane dialysis bags and suspended in 20 ml. of saline at 10 C. for 48 hours. At the termination of dialysis the volumes inside and outside the bags were recorded and penicillin assays were made on the serum within and on the saline

fraction outside the tubing. Quadruplicate experiments were run. Table II summarizes the results of these experiments.

The binding values for penicillins G and V are in close agreement with those of Anderson et al.⁵ The results with potassium α -phenoxyethyl penicillin indicate perhaps a slightly greater affinity for protein than was observed with penicillin V. Both penicillin V and α -phenoxyethyl penicillin possess greater protein affinities than penicillin G.

Serum Elimination. Serum disappearance curves following intravenous administration of 10 mg./Kg. of potassium α -phenoxyethyl penicillin and potassium penicillin V to unanesthetized dogs possessed similar slopes indicating that these animals eliminated the two penicillins from the serum at comparable rates. Figure 6 presents the results obtained.

DISCUSSION

Acute toxicity studies in mice and dogs have demonstrated that potassium α -phenoxyethyl penicillin has a very low toxicity. It is no more toxic acutely than potassium penicillin G or V. Chronic oral administration of enormous doses of the new penicillin to rats and dogs resulted in no cumulative toxicity as measured by observation, weight, appetite, and complete laboratory workup over an extended period. The compound possesses no measurable irritant liability as measured by a number of tests and therefore would not be expected to produce gastrointestinal distress due to local mucosal irritation when taken orally nor to be painful if injected intramuscularly.

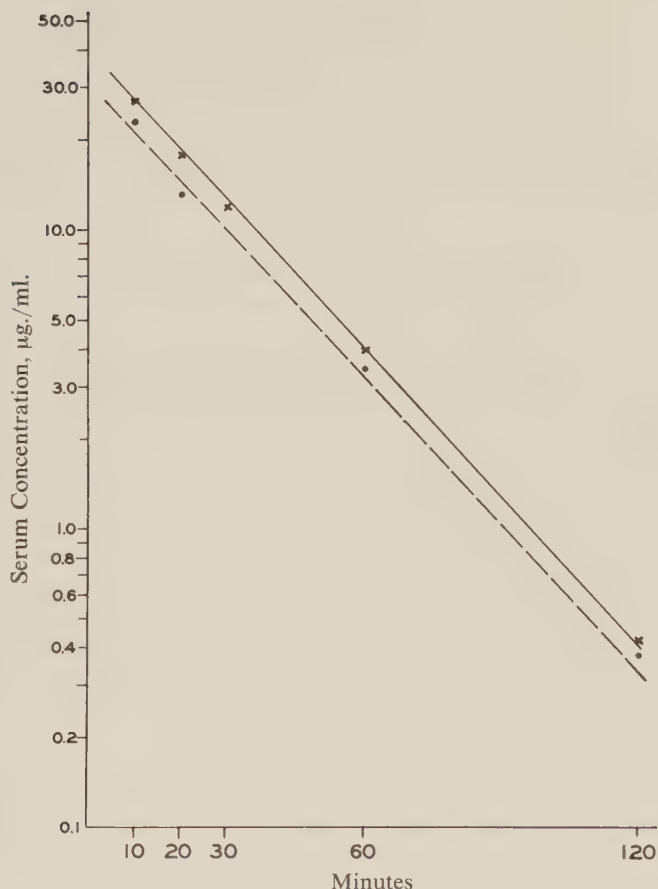
No significant cardiovascular or central nervous system effects occurred at reasonable doses.

α -Phenoxyethyl penicillin was more efficiently and rapidly absorbed than peni-

TABLE II
Serum Binding of Penicillins

Penicillin	Experiment no.	Serum bound, %
Potassium α -phenoxyethyl	1	41.9
	2	46.2
	3	41.4
	4	39.9
Mean		42.3
Potassium G	1	27.2
	2	24.3
	3	30.3
	4	29.4
Mean		27.8
Potassium V	1	33.5
	2	32.9
	3	37.2
	4	38.3
Mean		35.5

FIG. 6. Serum disappearance curves in dogs following intravenous administration of the same dose (10 mg./Kg.) of potassium α -phenoxyethyl penicillin and potassium penicillin V. —, potassium α -phenoxyethyl penicillin; - - -, potassium penicillin V.



cillin V following oral administration as evidenced by superior serum concentrations of the former 30 minutes and one hour after administration to fasted dogs.

The observation that similar peak serum levels were obtained in dogs given equal doses of α -phenoxyethyl penicillin by oral and intramuscular routes indicates that this penicillin is as efficiently absorbed from the gut as from muscle in contrast to the relatively poorer oral absorption of penicillin G.

Absorption of α -phenoxyethyl penicillin from the stomach, duodenum, and ileum proceeded rapidly in that order of increasing absorptive capacity. The observation that ileal absorption of α -phenoxyethyl penicillin surpassed duodenal absorption was unexpected but was found to be the case with potassium penicillin V also in contrast to previous authors²⁴ work on sodium penicillin G.

Serum binding studies demonstrated roughly comparable protein affinities for α -phenoxyethyl penicillin and penicillin V with perhaps slightly more binding of the former. The slight difference in protein affinity between the two penicillins would not be expected to be of practical significance.

Assuming that similar serum disappearance rates for α -phenoxyethyl penicillin and penicillin V indicate comparable net rates of tissue distribution, excretion, and metabolism for the two compounds, the differences in serum concentrations observed following oral administration to animals must therefore be due to differences in absorption characteristics.

SUMMARY

1. Potassium α -phenoxyethyl penicillin is no more acutely toxic than potassium penicillin G.

2. Rats and dogs tolerated daily administration of large oral doses of the new penicillin for over 12 weeks without behavioral, visceral, or hematopoietic alterations.

3. Potassium α -phenoxyethyl penicillin provided higher serum concentrations in dogs than potassium penicillin V when both were administered orally.

4. Oral absorption of potassium α -phenoxyethyl penicillin was similar to absorption from muscle.

5. The new penicillin was rapidly absorbed from all parts of the gastrointestinal tract (colon, stomach, duodenum, and ileum in order of increasing absorptive capacity).

6. The serum protein affinity of potassium α -phenoxyethyl penicillin was approximately equivalent to that of potassium penicillin V.

7. Serum disappearance rates for potassium α -phenoxyethyl penicillin and potassium penicillin V were comparable indicating that major differences in rates of excretion, tissue distribution, and metabolism probably do not exist.

8. Higher serum levels attained following oral administration of potassium α -phenoxyethyl penicillin are therefore likely due to more efficient absorption of this compound than of potassium penicillin V.

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Clinical and Laboratory Studies with Potassium Penicillin-152 [Potassium (α -Phenoxyethyl)-Penicillin]: A New Synthetic Penicillin

E. M. E. MORIGI, W. B. WHEATLEY, AND H. ALBRIGHT

Bristol Laboratories Inc., Syracuse, N. Y.

Penicillin has been available for 16 years and its usefulness and limitations are well established. The appearance of resistant strains of *Staphylococcus aureus* and the increasing number of serious allergic manifestations represent the main drawbacks to its extensive and efficient use.

The making of new and different penicillins received marked impetus with the work of Sheehan and Henery-Logan¹ who succeeded in accomplishing the first synthesis of a natural as well as related penicillins. Similarly the isolation of 6-aminopenicillanic acid by Batchelor et al⁴ and subsequent methods of production gave great impetus to the synthesis and evaluation of numerous new and different penicillins. This led to the development of a new penicillin, potassium α -phenoxyethyl penicillin.²

Since its development, potassium α -phenoxyethyl penicillin has been subjected to extensive microbiological³ and pharmacological⁵ studies. Clinical studies on absorption, excretion, toxicity, tolerance, and efficacy will be presented in this report.

MATERIALS AND METHODS

The absorption, excretion, toxicity, and tolerance studies were performed on healthy volunteers. Potassium α -phenoxyethyl penicillin was used mainly in two dosage forms, 250 and 500 mg. The drug was administered exclusively by mouth in a tablet form containing 250 mg. of potassium α -phenoxyethyl penicillin.

The bioassay studies were performed by the use of the plate diffusion method using *Sarcina lutea* as the test organism. Ten healthy subjects received one tablet of potassium α -phenoxyethyl penicillin 1 hour before meals and blood samples were withdrawn after 0, 1, 2, 4, and 6 hours. In a comparative study with 250 mg. per tablet of potassium penicillin V an identical procedure was employed.

From a large group of volunteers, 210 healthy individuals were chosen after transaminase, blood urea nitrogen, thymol turbidity, and complete blood tests were found to be within normal limits. These subjects were divided into three groups.

Group one consisted of 150 men who received one tablet of potassium α -phenoxyethyl penicillin, 250 mg., four times a day for 15 days. Group two was made up of 50 men who received two tablets of potassium α -phenoxyethyl penicillin, 500 mg., four times a day for 15 days. Group three consisted of 10 individuals who received one tablet of potassium α -phenoxyethyl penicillin, 250 mg., four times a day for 21

The trade name of Bristol Laboratories Inc. for potassium α -phenoxyethyl penicillin is Syncillin.

TABLE I

Average Serum Concentrations of 10 Volunteers in $\mu\text{g.}$ after a Single 250 mg. Oral Dose of Potassium α -Phenoxyethyl Penicillin and Potassium Penicillin V One Hour before Meals

Drug	Hours				
	0	1	2	4	6
Potassium α -phenoxyethyl penicillin	0	3.46	.46	.15	.02
Potassium penicillin V	0	1.62	.40	.05	<.01

days. The 210 volunteers had the previously mentioned laboratory tests repeated weekly until the end of the study.

The subjects observed for toxicity were also studied for their ability to tolerate this new antibiotic. These volunteers were checked daily for any complaints relating to the various body systems.

Forty-seven patients were used for the efficacy studies with potassium α -phenoxyethyl penicillin. Their disease states ranged from infections of the upper respiratory tract and ear to secondary infections of the skin. Whenever possible, the causative organism was isolated culturally before and after treatment.

RESULTS

Absorption Studies. In the first experiment, the absorption of potassium α -phenoxyethyl penicillin by the gastrointestinal tract was studied by administering one tablet, 250 mg., of the drug one hour before meals. Bloods were withdrawn at 0, 1, 2, 4, and 6 hours. The same procedure was repeated one week later with the administration of one tablet of potassium penicillin V. The average values of the blood levels obtained are shown in table I and figure 1.

Figure 1 shows that peak concentrations are obtained about one hour after the administration of potassium α -phenoxyethyl penicillin and that the serum concentrations of potassium α -phenoxyethyl penicillin are twice as high as those obtained with potassium penicillin V in the first hour. The serum concentrations decrease below therapeutic levels in approximately four hours. The levels of potassium

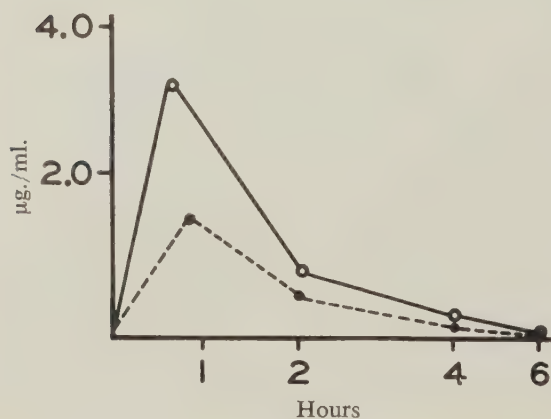


FIG. 1. Penicillin serum concentrations following a single oral 250 mg. dose. — — — Potassium penicillin V; ————— potassium α -phenoxyethyl penicillin.

TABLE II

Average Serum Concentrations ($\mu\text{g./ml.}$) of 20 Volunteers after a Single 250 mg. Oral Dose of Potassium α -Phenoxyethyl Penicillin and Potassium Penicillin V One Hour before Meals

Drug	Hours						
	0	1/2	1	2	4	6	8
Potassium α -phenoxyethyl penicillin	0	3.72	3.83	.89	.18	.01	0
Potassium penicillin V	0	1.69	1.55	.79	.17	.02	0

α -phenoxyethyl penicillin are generally more maintained than potassium penicillin V in the first four hours.

Serum concentrations with 20 volunteers in another crossover study with potassium α -phenoxyethyl penicillin and potassium penicillin V gave much the same results. Table II shows that potassium α -phenoxyethyl penicillin gives serum levels twice as high as potassium penicillin V.

The urines of 10 healthy individuals, after the administration of 250 mg. of potassium α -phenoxyethyl penicillin, were collected at six hour intervals and an aliquot assayed. From these data, the amount and rate of excretion of potassium α -phenoxyethyl penicillin were calculated. An identical experiment was performed with 250 mg. of potassium penicillin V one week later.

Table III summarizes the average urine concentrations of the 10 volunteers and compares the cumulative urinary excretion of potassium α -phenoxyethyl penicillin with potassium penicillin V.

The higher percentage of potassium α -phenoxyethyl penicillin excreted is another indication of the superior absorption of this penicillin in man. From table III it is evident that most of the potassium α -phenoxyethyl penicillin is excreted in the first six hours and in the next 12 and 24 hours the excretion is almost nil.

Toxicity. Potassium α -phenoxyethyl penicillin was given to 210 healthy volunteers in tablet form, 250 mg. For 15 days, 150 of them received one tablet four times a day, 50 received two tablets four times a day for 15 days, and 10 received one tablet four times a day for 21 days. These individuals had their blood samples withdrawn at weekly intervals and tested for any change in transaminase (Sigma-Frankel 8 to 40 units), thymol turbidity (MacLagen 0.4 to 4.0 units), and blood

TABLE III

Average Urine Concentrations in mg. after a Single 250 mg. Dose of Potassium α -Phenoxyethyl Penicillin and Potassium Penicillin V

Drug	Hours					
	0 to 6		6 to 12		12 to 24	
	mg.	%	mg.	%	mg.	%
Potassium α -phenoxyethyl penicillin	77.3	30.9	1.0	0.4	0	0
Potassium penicillin V	45.7	18.2	0.7	0.2	0	0

TABLE IV

Bacteriological Response of Microorganisms Isolated from Clinical Cases

	No. isolates	Response	
		Satisfactory (elimination of organism)	Unsatisfactory (persistence of organism)
<i>Staphylococcus aureus</i>	20	19	1
<i>Streptococcus hemolyticus</i>	7	7	

urea nitrogen (Karr 12 to 15 mg.). It was observed that during this study the transaminase, blood urea nitrogen, and thymol turbidity fell within normal limits. The white and red cell counts and hemoglobin were checked weekly during this study. Each showed a wide variation during each observation period and between each period. By the F test of variances it was found that there was no significant difference between the variability of the control period ($\sigma = 1.83$), one week ($\sigma = 1.75$), and two weeks ($\sigma = 1.93$). If the mean differences in the white blood cell counts between each observation period are analyzed, there is no evidence of a difference in relation to the control. Additional tests on the observations in the 10 subjects studied for three weeks revealed similar findings.

Efficacy. Forty-seven patients with different infections, most of which were proved bacteriologically, were included in this study. These infections ranged from acute infections of the upper respiratory tract and ear to secondary infections of the skin due to *Staph. aureus*.

Therapy was by the oral route and the total amounts of potassium α -phenoxyethyl penicillin administered varied from a minimum of 3 Gm. to a maximum of 7 Gm. on a regimen of four tablets (1 Gm.) a day. Of these patients, 38 were cured, 6 improved, and in 3 patients no change was noted. Twenty strains of *Staph. aureus* were isolated by culture prior to initiation of treatment. Nineteen strains were found to be highly sensitive to potassium α -phenoxyethyl penicillin and one

TABLE V

Clinical Studies: Number of Patients Diagnosed and Treated with Potassium α -Phenoxyethyl Penicillin

Diagnosis	No. of patients	Average duration of treatment in days
Acute tonsillitis	3	5
Otitis externa	1	4
Otitis media	2	5
Acute pharyngitis	3	5
Cellulitis	5	5
Furunculosis	8	5
Carbuncle	1	6
Thrombophlebitis	2	5
Pyoderma	18	5
Impetigo	1	3
Abscess	2	6
Vincent's angina	1	5

TABLE VI

Minimum Inhibitory Concentrations of Penicillins G, O, V and Potassium (α -Phenoxyethyl) Penicillin against Recent Isolates of Coagulase-Positive Staph. aureus

	Resistant, 15.0 μ g. or more	Moderately resistant, 3.9–7.8 μ g.	Sensitive, 2.0 μ g. or less
Penicillin G	61	19	24
Penicillin O	58	23	29
Penicillin V	53	22	29
Potassium α -phenoxyethyl penicillin	40	14	50

resistant. Seven strains of β -hemolytic *Streptococcus*, the majority of which were isolated from the upper respiratory tract, were found to be sensitive.

Table IV indicates the bacteriological responses observed following oral administration of potassium α -phenoxyethyl penicillin.

Table V shows the diagnoses of patients treated with potassium α -phenoxyethyl penicillin.

None of the 210 healthy volunteers had any complaints that might be representative of side action liability.

The minimum inhibitory concentrations of potassium α -phenoxyethyl penicillin, penicillin G, penicillin O, and penicillin V were determined for 104 strains of *Staph. aureus*. The serial tube dilution technique was employed using Trypticase soy broth. It was found that potassium α -phenoxyethyl penicillin inhibited 50 strains at 2.0 μ g. or less, whereas the other penicillins inhibited fewer strains. Table VI shows the number of strains resistant, moderately resistant, and sensitive to penicillin G, penicillin O, penicillin V, and potassium α -phenoxyethyl penicillin.

From table VI it is possible to note that potassium α -phenoxyethyl penicillin is more active than penicillins G, O, and V in inhibiting coagulase-positive *Staph. aureus*.

SUMMARY AND CONCLUSIONS

The oral administration of potassium α -phenoxyethyl penicillin (potassium penicillin-152) yields higher and more constant blood level concentrations than potassium penicillin V. The results described for both blood and urine concentrations indicate that absorption of potassium α -phenoxyethyl penicillin from the alimentary tract is twice that of potassium penicillin V in the first hour. The urinary excretion studies show also that potassium α -phenoxyethyl penicillin is more completely excreted for any given dose. In comparison with potassium penicillin V, a greater percentage of potassium α -phenoxyethyl penicillin is excreted.

The clinical results indicate that this new penicillin is effective in the treatment of infections due to *Staph. aureus* and β -hemolytic streptococci. It is apparent that infections of the upper respiratory tract, middle and external ear, and skin when caused by susceptible microorganisms respond readily to treatment with potassium α -phenoxyethyl penicillin.

The minimum inhibitory concentration values on 104 strains of *Staph. aureus* obtained in a comparative study with three other penicillins denote that potassium α -phenoxyethyl penicillin inhibits a greater number of penicillin G resistant *Staph. aureus* organisms than other penicillins, namely, penicillins O and V. These studies indicate that a greater number of bacterial infections caused by *Staph. aureus* may respond to treatment with potassium α -phenoxyethyl penicillin. However, conclusive evidence must await additional clinical study. The greatly improved absorption of this new penicillin seems to increase the possibility of this latter being true. The rapidity of excretion points out that there is no danger of accumulation. No evidence of intolerance or allergic phenomena have been observed in these 257 individuals.

ACKNOWLEDGMENT

We want to express our appreciation to Dr. William Wright of the Food and Drug Administration and to Dr. A. M. Kligman, University of Pennsylvania, Philadelphia, Pa., for allowing us to include their information on potassium α -phenoxyethyl penicillin studies in this paper.

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Laboratory and Clinical Studies with Potassium Penicillin-152 [Potassium (α -Phenoxyethyl)-Penicillin]

G. A. CRONK, D. E. NAUMANN, H. ALBRIGHT, AND W. B. WHEATLEY

*Department of Health and Preventive Medicine, Syracuse University, and
Bristol Laboratories Inc., Syracuse, N. Y.*

Potassium α -phenoxyethyl penicillin* (potassium penicillin-152) was produced synthetically by Perron and co-workers¹ in 1959. It is the N-acylation product of 6-aminopenicillanic acid and α -phenoxypropionic acid. In this paper, the mixture of diastereoisomers derived from this reaction is called α -phenoxyethyl penicillin. The pure diastereoisomers have also been obtained by using D α -phenoxypropionic acid and L α -phenoxypropionic acid.¹ These will be referred to as D- and L-isomers. Preliminary studies demonstrated this antibiotic to be very soluble in water, resistant to acid decomposition, readily absorbed from the gastrointestinal tract, and similar to other penicillins in its in vitro antibacterial spectrum.^{2,3}

The absorption of the potassium salt of this penicillin and the rate of its excretion in the urine were studied in normal human beings. The data from these experiments and some observations on this antibiotic's use in infections will be presented in this paper.

METHODS AND MATERIALS

Potassium α -phenoxyethyl penicillin exists as two isomers. Both of these were studied in their pure forms and in a mixture as produced in routine plant synthesis. Tablets containing 268 mg. of the antibiotic were used in this study. Healthy adults between the ages of 21 and 55 years were employed in the absorption and excretion studies.

The first set of experiments was designed to determine the penicillin serum concentrations after single doses of the antibiotic. These were performed with volunteers who had been fasting for seven or more hours. Blood samples were withdrawn at 0, $\frac{1}{2}$, 1, 2, and 4 hours and the serum was assayed for penicillin content using *Sarcina lutea*, FDA 1001, as the test organism. In an attempt to correlate serum penicillin levels with the dose of phenoxyethyl penicillin administered, doses ranging from 134 to 2144 mg. were studied in the manner described.

In the second set of experiments, the penicillin serum levels were determined following repeated administrations of 268 and 536 mg. every six hours and every 12 hours. In the six hour replacement study, the antibiotic was administered in varying time relationships to meals; in the 12 hour replacement study, the first, third, and fifth doses were administered fasting, while the second, fourth, and sixth doses were administered one hour after the evening meal. During these tests, all urine excreted was collected in two hour or six hour units and assayed for penicillin content.

In order to determine more readily the effect of food ingestion on the absorption of potassium α -phenoxyethyl penicillin, a third set of experiments was performed.

* The trade name of Bristol Laboratories Inc. for potassium α -phenoxyethyl penicillin is Syncillin.

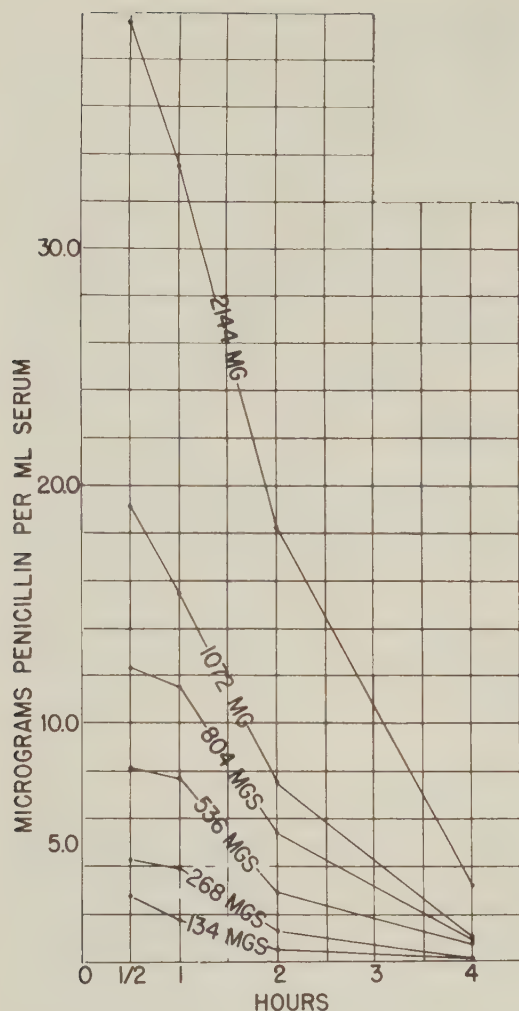


FIG. 1. Average concentration of penicillin in serum following oral administration of various doses of potassium α -phenoxethyl penicillin.

Five groups, each composed of 10 volunteers, were used. A dose of 268 mg. was administered as follows: Group I, 60 minutes before food; group II, 30 minutes before food; group III, 15 minutes before food; group IV, with food; and group V, 60 minutes after food. The food consumed consisted of one cup of soup, one meat sandwich with mayonnaise and butter, and one glass of milk. Blood samples were withdrawn at one half, one, and two hours after administration of the antibiotic.

Patients seen in the University Infirmary with various types of infections were treated with either 268 or 536 mg. of potassium α -phenoxethyl penicillin every four to six hours. Standard bacteriological and hematological studies were performed on each patient.

Single Dose Experiments. The average penicillin serum concentrations following the oral administration of potassium α -phenoxethyl penicillin in single doses ranging from 134 to 2144 mg. are presented in figure 1 and table I. After the completion of this set of experiments, several things were evident. First, this antibiotic is absorbed rapidly from the fasting gastrointestinal tract and reaches its highest concentration in the blood approximately one half hour after administration.

TABLE I

Average Penicillin Serum Concentrations ($\mu\text{g./ml.}$) after the Oral Administration of Various Doses of Potassium α -Phenoxyethyl Penicillin

Dose, mg.	Number of volunteers	0 hour	1/2 hour	1 hour	2 hours	4 hours
134	20	0	2.72 (1.2-5.9)	1.74 (0.7-4.1)	0.50 (0.2-2.4)	0.08 (0.4-0.15)
268	105	0	4.28 (0.2-8.4)	3.89 (0.8-10.0)	1.26 (0.3-6.0)	0.10 (0-1.2)
536	25	0	8.15 (2.5-23.0)	7.77 (2.2-17.0)	2.91 (0.8-5.6)	0.32 (0.2-1.4)
804	15	0	12.3 (0.9-24.0)	11.5 (2.7-17.0)	5.40 (2.2-10.0)	0.82 (0.3-1.2)
1072	15	0	19.1 (2.7-24.0)	15.4 (6.2-27.0)	6.45 (3.4-12.0)	1.02 (0.4-1.7)
2144	5	0	39.6 (25-65)	33.6 (26-47)	18.2 (10-43)	3.16 (1.5-8.5)

Second, there is a fairly rapid penicillin run-off; penicillin serum levels measured at one hour ranged between 63 and 95 per cent of the one half hour levels, while at two hours they ranged between 18 and 46 per cent, and at four hours they ranged between 2 and 8 per cent. Third, when the average penicillin serum levels for any one time period are plotted as a function of the amount of antibiotic administered, the resulting curves are essentially straight lines (fig. 2). Therefore, it appears that the average amount of penicillin in the serum can be predicted with reasonable accuracy when a specific dose is administered to normal human beings in a fasting state.

A crossover comparison of penicillin serum levels following the oral administration of 250 mg. of potassium phenoxymethyl penicillin and 268 mg. of potassium α -phenoxyethyl penicillin to 25 subjects revealed somewhat higher serum levels with the latter antibiotic (fig. 3).

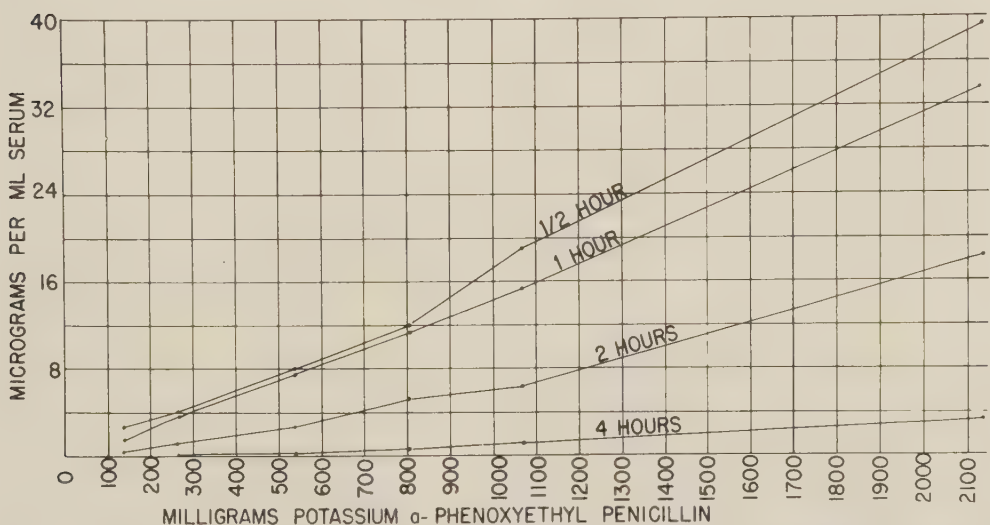


FIG. 2. Average penicillin serum concentrations at specific times in relationship to the oral administration of various doses of potassium α -phenoxyethyl penicillin.

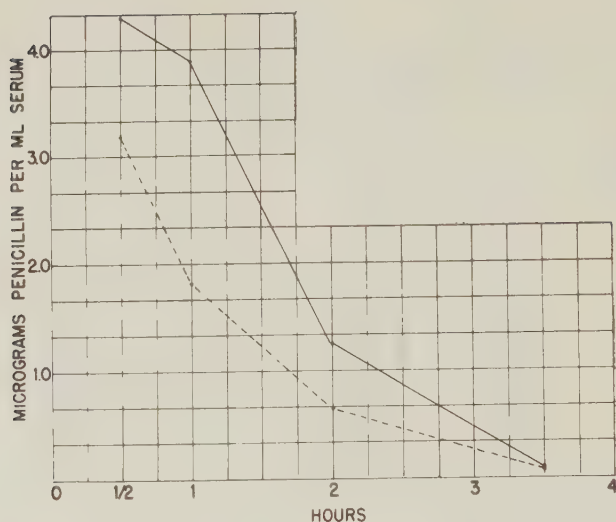


FIG. 3. A comparison of average penicillin serum concentrations in a crossover study of potassium α -phenoxymethyl penicillin and potassium phenoxymethyl penicillin (25 subjects). —, 268 mg. potassium α -phenoxymethyl penicillin; ---, 250 mg. potassium phenoxymethyl penicillin.

Because of the problems inherent in having an antibiotic exist in more than one isomeric form, a 268 mg. dose of each of the pure diastereoisomers of α -phenoxymethyl penicillin and a mixture of the two (70 per cent L and 30 per cent D) was given to groups of 10 volunteers. The subsequent antibiotic serum levels are presented in table II and figure 4. The one hour levels were essentially the same for all three products. At two hours, the blood levels with the L-isomer and the mixture were double those obtained with the D-isomer. The difference in concentrations continued to be apparent at four hours. The mixture of the two isomers produced blood levels almost identical to a prediction that was based on the levels obtained from the pure isomers.

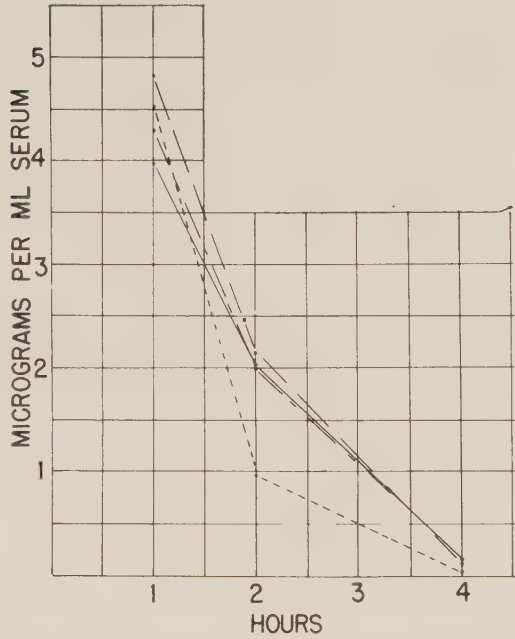
Multiple Dose Experiments. After completing the single dose experiments with potassium α -phenoxymethyl penicillin, multiple dose experiments were designed to examine the more practical aspects of antibiotic therapy, such as the maintenance of antibiotic serum concentrations. In the first of these tests, 268 mg. of the antibiotic was administered every four hours to a group of 10 subjects. The average penicillin serum levels at one and four hours are presented in figure 5. The first dose was given at 7:00 a.m. after the volunteers had fasted for seven or more hours. The subsequent doses given at four hour intervals had varying time relationships with the daily meals (breakfast at 9 a.m., lunch at 12 noon, and dinner at 5 p.m.). The average one hour penicillin serum level after the first dose was 5.24 $\mu\text{g./ml.}$ while the subsequent one hour levels were 3.15, 2.64, 1.89, and 3.6

TABLE II

Average Penicillin Serum Concentrations ($\mu\text{g./ml.}$) after the Oral Administration of the Pure Isomers of Potassium α -Phenoxymethyl Penicillin and Mixtures of the Isomers

Product	Dose, mg.	1 hour	2 hours	4 hours
L-isomer	268 mg.	4.05	2.12	0.19
D-isomer	268 mg.	4.52	0.97	0.05
D-isomer, 30%, and L-isomer, 70%	268 mg.	4.33	2.00	0.15

FIG. 4. Average penicillin serum concentrations following the oral administration of 268 mg. of a mixture of levo- and dextro-potassium α -phenoxyethyl penicillin in comparison with 268 mg. of the pure isomers. —, 268 mg. levo-potassium α -phenoxyethyl penicillin; - - - - , 268 mg. dextro-potassium α -phenoxyethyl penicillin; — — —, 268 mg. 70 per cent levo- and 30 per cent dextro-potassium α -phenoxyethyl penicillin; — — — —, 268 mg. 70 per cent levo- and 30 per cent dextro-potassium α -phenoxyethyl penicillin.



$\mu\text{g./ml.}$ The last serum level was obtained when the antibiotic was administered six hours after the evening meal and the intervening lower levels resulted when the antibiotic was given in close relationship to meals. It appears that the presence of food in the stomach or upper gastrointestinal tract has a definite effect on the absorption of potassium α -phenoxyethyl penicillin.

Three additional multiple dose experiments were performed using groups of 10 volunteers. In the first of these, 268 mg. was given every six hours and in the second, every 12 hours; in the third, 536 mg. was given every 12 hours. In

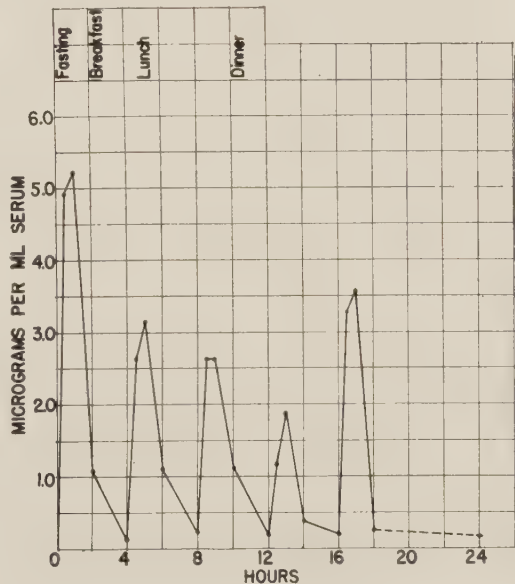


FIG. 5. Average serum concentrations following the oral administration of 268 mg. of potassium α -phenoxyethyl penicillin every four hours.

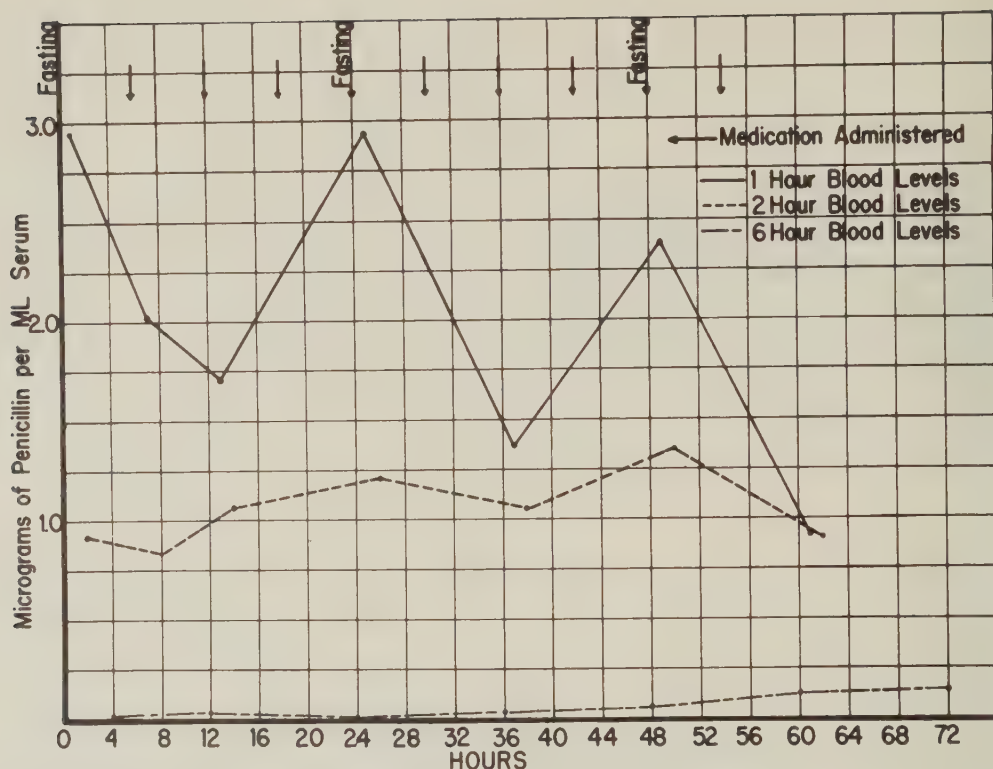


FIG. 6. Average penicillin serum concentrations following the oral administration of 268 mg. of potassium α -phenoxyethyl penicillin every six hours.

addition to establishing serum antibiotic concentration patterns, these tests made the relationship of blood levels to food intake again evident (figs. 6, 7, 8 and table III). During the 12 hour interval experiments, the morning dose was always administered after the subjects had fasted for seven or more hours and the fast was maintained for two hours after ingestion of the antibiotic. The evening dose was

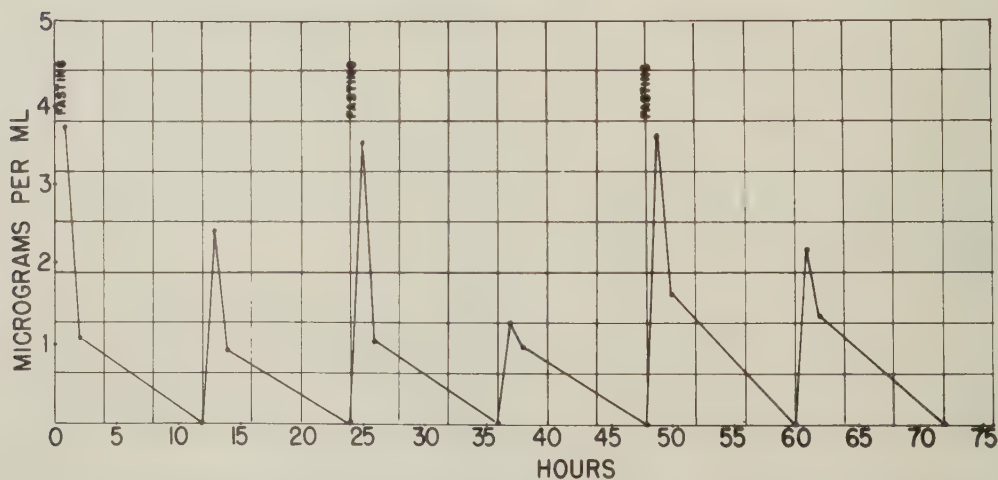


FIG. 7. Average penicillin serum concentrations following oral administration of 268 mg. of potassium α -phenoxyethyl penicillin every 12 hours. Alternating doses administered fasting.

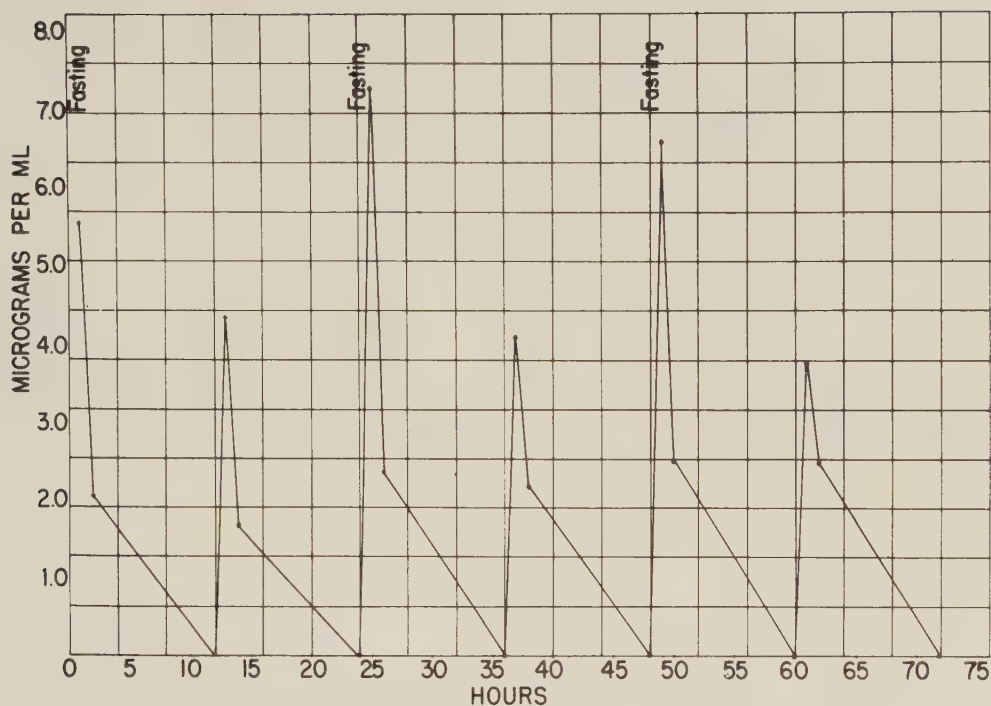


FIG. 8. Average penicillin serum concentrations following the oral administration of 536 mg. of potassium α -phenoxyethyl penicillin every 12 hours.

TABLE III

Average Penicillin Serum Concentrations (μ g./ml.) after the Oral Administration of Potassium α -Phenoxyethyl Penicillin in Repeated Doses

Hour	268 mg. every 6 hours (10 patients)	268 mg. every 12 hours (10 patients)	536 mg. every 12 hours (10 patients)
0	0	0	0
1	2.96	3.73	5.55
2	0.89	1.07	2.04
4	0.02		
7	2.03		
8	0.85		
12	0.04	0	0
13	1.71	2.41	4.31
14	1.07	0.91	1.67
24	0.02	0	0
25	2.94	3.54	7.28
26	1.21	1.03	2.37
36	0.04	0	0
37	1.37	1.26	4.10
38	1.06	0.95	2.17
48	0.07	0	0
49	2.39	3.62	6.61
50	1.36	1.62	2.51
60	0.14	0	0
61	0.96	2.20	3.75
62	0.95	1.35	2.48
72	0.15	0	0

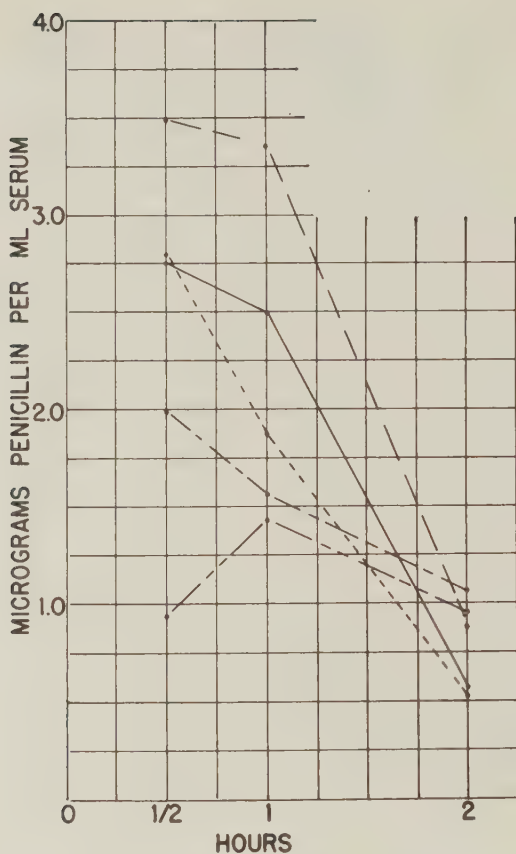


FIG. 9. The effect of food on the absorption of potassium α -phenoxethyl penicillin. —•—, 268 mg. 60 minutes before food; - - -•-, 268 mg. 30 minutes before food;•-, 268 mg. 15 minutes before food; — — —•-, 268 mg. with food; — • —•-, 268 mg. 60 minutes after food.

given one hour after eating a full dinner. In every instance, the one hour penicillin serum levels were much higher when the antibiotic was administered during the fasting state. In all of these experiments, there was no evidence of the retention of the antibiotic more than four hours after its administration. As would be expected, there was no antibiotic build-up with repetitive doses at 4, 6, or 12 hour intervals.

TABLE IV

The Relation of Food Intake to the Average Penicillin Serum Concentrations ($\mu\text{g./ml.}$) Obtained after the Oral Administration of Potassium α -Phenoxethyl Penicillin, 268 mg.

	Number of volunteers	1/2 hour	1 hour	2 hours
Medication 60 minutes before food	10	3.48 (1.4–5.8)	3.33 (1.8–4.6)	0.87 (0.5–1.3)
Medication 30 minutes before food	9	2.76 (0.6–5.5)	2.50 (1.0–3.8)	0.57 (0.3–1.0)
Medication 15 minutes before food	10	2.79 (0.8–4.7)	1.89 (1.3–2.9)	0.54 (0.3–0.8)
Medication with food	9	1.99 (0.7–3.9)	1.58 (0.6–2.6)	1.09 (0.5–1.9)
Medication 60 minutes after food	10	0.93 (0.5–2.4)	1.42 (0.7–3.4)	0.96 (0.4–1.8)

In the experiments described at this point, it has been seen that the ingestion of food has more than a casual effect on the antibiotic serum levels. To investigate this point further, a dose of 268 mg. of potassium α -phenoxyethyl penicillin was given to five groups of volunteers at various time relationships to the ingestion of food. These results are presented in figure 9 and table IV. The highest one half hour and one hour serum levels were obtained when the antibiotic was given one hour before food ingestion. There was then a gradual quantitative shift to the lowest serum levels that occurred when the penicillin was given one hour after eating. It is of some interest to note that while the serum levels are lower when

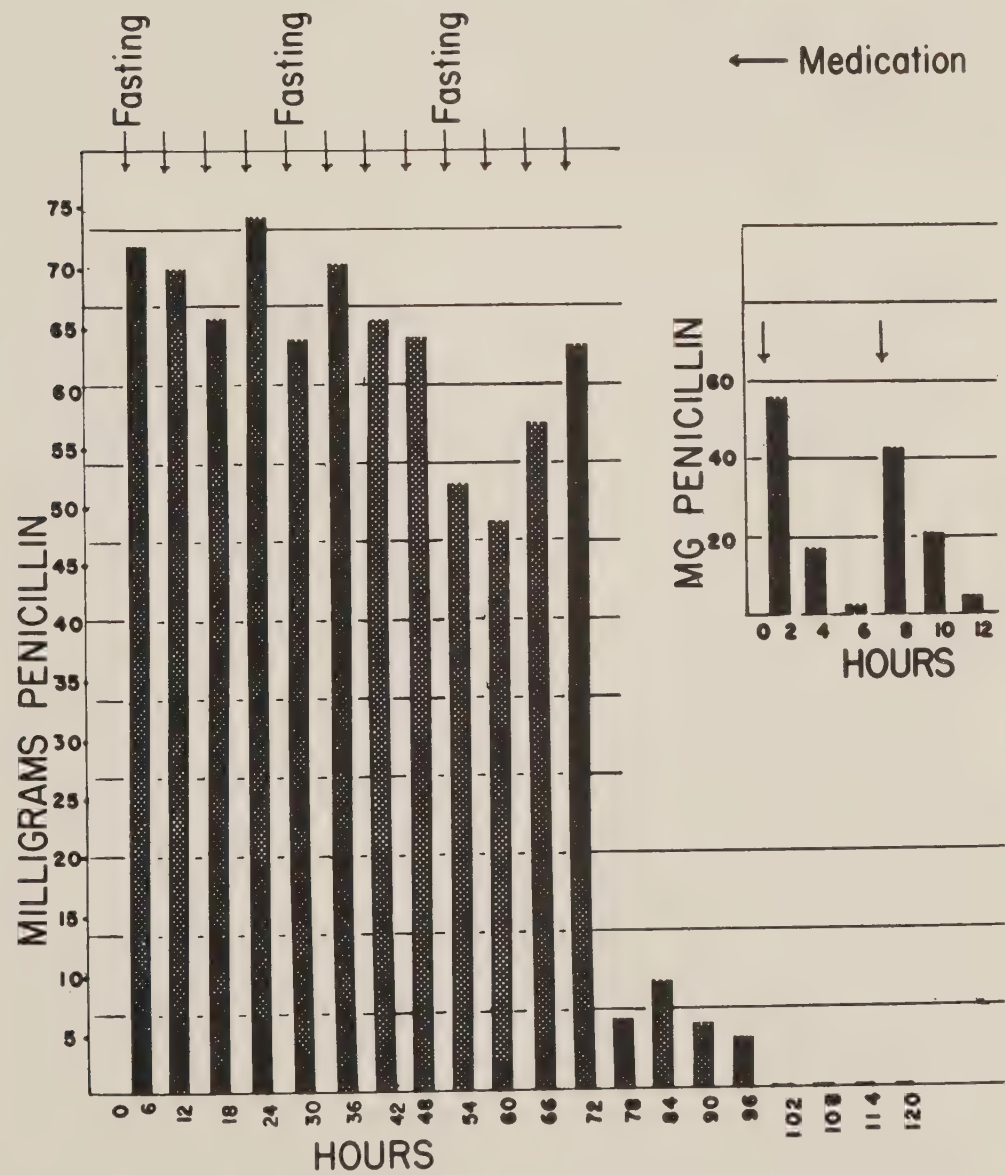


FIG. 10. Average amount of penicillin found in urine following the oral administration of potassium α -phenoxyethyl penicillin every six hours.

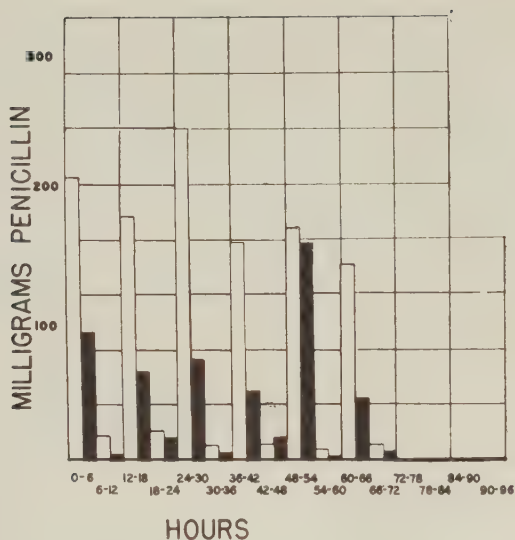


FIG. 11. Average excretion of penicillin in urine following oral administration of 536 mg. and 268 mg. of potassium α -phenoxyethyl penicillin every 12 hours. \square , 536 mg. potassium α -phenoxyethyl penicillin every 12 hours; \blacksquare , 268 mg. potassium α -phenoxyethyl penicillin every 12 hours.

the antibiotic is given with or after meals, the curves produced by these levels had a tendency to be horizontal when compared with the other curves.

Excretion. Several experiments were performed to study the excretion of penicillin in the urine following the oral administration of potassium α -phenoxyethyl penicillin. In the first of these, all urine passed was collected in two hour units following the administration of two doses (six hours apart) of 268 mg. The first dose was given to subjects in a fasting state and the second dose was administered one hour after lunch. The average amount of penicillin excreted is presented in figure 10. Approximately 28 per cent of the first dose was identified in the urine during the six hours after administration and about 25 per cent of the second dose was identified during the next six hours. Of the total amount of antibiotic excreted during the two six hour periods, 73 and 62 per cent, respectively, was excreted during the first two hours, 23 and 30 per cent during the second two hours, and 3 and 6 per cent during the third two hours. This experiment was then extended by the continued administration of 268 mg. every six hours for 72 hours. During this period, urine was collected in six hour units. Approximately the same amount of penicillin was excreted in each of the subsequent six hour periods; 790.2 mg., or 24 per cent, of the total penicillin administered (3216 mg.) was found in the urine during the test period.

In the second set of experiments, penicillin doses of 268 and 536 mg. were administered every 12 hours for six doses. All urine was collected in six hour units and assayed for penicillin content. These results are presented in figure 11 and table V. During the first six hours after administration, an average of 90.5 (33 per cent) of the 268 mg. dose and 203 (37 per cent) of the 536 mg. dose was identified in the urine. During the second six hours, urine assay showed 1.6 and 3 per cent of the respective doses. When the entire experiment was reviewed, it became apparent that the amount of penicillin recovered in each six hour sample reflected the fasting or nonfasting state of the subjects. More penicillin was recovered in the urine during the fasting state than during the nonfasting state. This correlates well with the penicillin serum levels obtained in the same experiment,

which were presented previously. A total of 1608 mg. of phenoxyethyl penicillin was given in 268 mg. doses and 3216 mg. in 536 mg. doses; 532.5 mg., or 33 per cent, of the former was identified in the urine during the test period and 1149.6 mg., or 37 per cent, of the latter was identified during the same period.

CLINICAL STUDIES

Thirty-eight patients with acute tonsillitis, gingivitis, otitis media, pneumonia, peritonsillar abscess, gonorrhea, cellulitis, conjunctivitis, and acute respiratory disease were treated with potassium α -phenoxyethyl penicillin in doses of 268 and 536 mg. In all cases in which a penicillin-susceptible bacterium was isolated (i.e., hemolytic *Streptococcus*, fusospirochetes, gonococcus, or pneumococcus), there was a prompt regression of the symptoms and pathology. Further experience undoubtedly will demonstrate that oral phenoxyethyl penicillin is a highly successful antibiotic in all infections caused by phenoxyethyl penicillin-sensitive organisms.

TOXICITY

Up to this time, side reactions resulting from the oral administration of potassium α -phenoxyethyl penicillin seem to be rare (table VI). One reaction did occur early in our experience, which might be attributed to the antibiotic. A man, 50 years of age who had recently returned from the Middle East, developed a typical streptococcal tonsillitis with fever and a polymorphonuclear leukocytosis. He was given 536 mg. of the penicillin every six hours. After 48 hours of therapy, his symptoms had disappeared and he was afebrile; the objective findings in the

TABLE V
Average Amounts (mg.) of Penicillin Recovered in the Urine after Repeated Oral Administrations of Potassium α -Phenoxyethyl Penicillin

Hour	268 mg. every 6 hours	268 mg. every 12 hours	536 mg. every 12 hours
0-6	71.5	90.5	203.0
6-12	70.0	4.3	16.4
12-18	65.4	64.7	165.0
18-24	74.5	16.9	23.0
24-30	64.1	73.5	237.0
30-36	70.3	5.9	10.7
36-42	65.4	50.0	155.0
42-48	64.6	16.8	11.5
48-54	51.3	153.5	165.0
54-60	48.9	3.5	8.0
60-66	56.6	44.7	142.0
66-72	63.4	8.2	13.0
72-78	5.9	0	0
78-84	9.3	0	0
84-90	5.4	0	0
90-96	3.6	0	0
Total excretion	790.2	532.5	1149.6
Total penicillin administered	3216	1608	3216
Per cent recovered	24	33	35

TABLE VI

Side Reactions with Potassium α -Phenoxyethyl Penicillin in Relation to the Number of Subjects and the Dose of Antibiotic

Single dose			Multiple dose		
Dose, mg.	No. of subjects	Toxicity	Total dosage, mg.	No. of subjects	Toxicity
134	20	0	Up to 1991	21	0
268	154	0	2000-3999	24	0
536	20	0	4000-5999	12	0
804	15	0	6000-7999	9	1
1072	15	0	8000-9999	6	0
2144	5	0	10,000 or more	5	0
4288	5	0			
Total	234	0		77	1

pharynx also had regressed toward normal. A throat culture taken at this time was negative for hemolytic *Streptococcus*. On the morning of the third day of therapy, the patient had a purpuric rash on his extremities, but otherwise was asymptomatic. A fragility test, the bleeding and clotting time, and a platelet count were normal. The antibiotic was changed to erythromycin propionate, which was continued for five days. During this time, the patient continued to be asymptomatic and the purpuric rash gradually disappeared. Four days after the cessation of antibiotic therapy, the patient developed another febrile episode that was associated with general malaise and a negative laboratory profile; this persisted for seven days. Two weeks later, a skin test with penicillin G and a passive transfer test were negative. In this rather complicated clinical picture, it was impossible to correlate positively the penicillin therapy with the purpuric rash.

SUMMARY AND CONCLUSIONS

1. Potassium penicillin-152 is a synthetic penicillin produced by the N-acylation of 6-aminopenicillanic acid and α -phenoxypropionic acid.

2. The absorption from the fasting gastrointestinal tract of potassium penicillin-152 in single doses ranging from 134 to 2144 mg. was studied. The penicillin serum levels at one half, one, two, and four hours were 2.72, 1.74, 0.5, and 0.08 $\mu\text{g./ml.}$ respectively after the ingestion of 134 mg.; and 39.6, 33.6, 18.2, and 3.16 $\mu\text{g./ml.}$ respectively following the ingestion of 2144 mg. The serum levels following intermediate doses of the antibiotic were directly proportional to the dose size.

3. The serum levels at one, two, and four hours after the ingestion of 268 mg. of the L-isomer of phenoxyethyl penicillin were 4.05, 2.12, and 0.19 $\mu\text{g./ml.}$ respectively; following 268 mg. of the D-isomer, the levels were 4.52, 0.97, and 0.05 $\mu\text{g./ml.}$ respectively.

4. A 268 mg. dose of potassium penicillin-152 administered every 4, 6, and 12 hours in a fasting state produced penicillin serum levels identical to the single dose experience. The presence of food in the upper gastrointestinal tract at the time of antibiotic administration interfered with penicillin absorption and the subsequent

serum levels were 30 to 60 per cent lower than those obtained during a fasting state. The same pattern occurred when 536 mg. was administered every 12 hours.

5. In order to obtain the best absorption of potassium penicillin-152 from the gastrointestinal tract, it should be administered in a fasting state or at least one hour before the ingestion of food.

6. Between 24 and 35 per cent of the doses of potassium penicillin-152 was identified in the urine during the first six hours after administration. Of the total excreted during this period, 62 to 73 per cent was excreted during the first two hours.

7. Thirty-eight patients with acute tonsillitis, gingivitis, otitis media, pneumonia, peritonsillar abscess, gonorrhea, conjunctivitis, and acute respiratory disease were treated successfully with potassium penicillin-152.

8. Two hundred and thirty-four subjects received a single dose of potassium penicillin-152 ranging from 134 to 4288 mg. without the occurrence of side reactions. Also, 77 subjects received multiple doses totaling from 1608 to 21,440 mg. One patient developed a purpuric rash after 6432 mg. of the penicillin had been administered over a period of three days.

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Interactions of a Synthetic Penicillin, 6-Benzylsulfonamidopenicillanic Acid, and Penicillinase-Producing Staphylococci

SIDNEY COHEN, LIPPMAN H. GERONIMUS, AND FELIX LEITNER

*Department of Microbiology, Michael Reese Hospital Medical Center, Chicago, Ill., and
Department of Laboratories, Beth Israel Hospital, Boston, Mass.*

The continuing problem of infections by antibiotic-resistant staphylococci has stimulated a search for new and more effective antibiotic agents. One approach has been the development of modifications of penicillin G by biosynthetic or chemical synthetic methods in the hope that the new compounds might be relatively resistant to staphylococcal penicillinase while retaining adequate antibiotic activity. The first chemically synthesized modified penicillin was the compound dl-6-benzylsulfonamidopenicillanic acid (benzylsulfonamido penicillin).¹ It differs from penicillin G in its side chain, which, in benzylsulfonamido penicillin, is linked to the penicillin ring structure by a sulfonyl group in place of the carbonyl group in penicillin G (fig. 1). An additional difference is that benzylsulfonamido penicillin is a racemate while penicillin G, a biosynthetic product, is a single enantiomorph. This paper reports some comparative studies of these two penicillins with respect to hydrolysis by staphylococcal penicillinase induction of staphylococcal penicillinase and ability to inhibit growth of penicillinase-producing staphylococci.

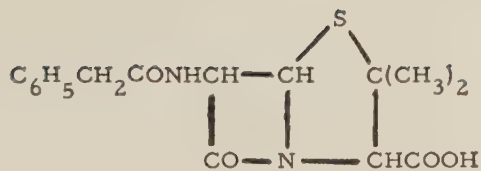
METHODS

The routine culture medium was a pancreatic digest of beef broth, either prepared at the Department of Bacteriology and Immunology, Harvard Medical School, or purchased as the dehydrated product (tryptic digest broth-Fields B.B.L.).

Staphylococcus aureus, strain 55-C-1, the routine test strain, is a penicillinase-producing *Staphylococcus* used in earlier studies.^{2,3} It produces relatively large amounts of penicillinase and in this respect is typical of many strains isolated from patients. *Staph. aureus* strains 1272 and 6549, also isolated from clinical material, are weaker penicillinase-producing strains with penicillinase activities approximately 1/5 to 1/10 that of strain 55-C-1. *Staph. aureus* strain 9 is a penicillin-sensitive strain and produces no detectable penicillinase.

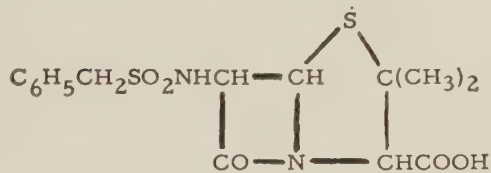
Penicillinase was assayed manometrically at 37 C. in 0.017 M sodium bicarbonate and in a gas phase of 5 per cent carbon dioxide—95 per cent N₂.⁴ Prior to assay staphylococci were washed with water or 0.017 M sodium bicarbonate by centrifugation and resuspended in 0.017 M sodium bicarbonate. To conserve our small supply of benzylsulfonamido penicillin, it was used as substrate in amounts limited to 4 to 18 μ m. This was dissolved in 0.2 ml. of 0.017 M sodium bicarbonate and tipped in from the sidearm. These amounts of substrate were adequate to saturate the available enzyme, as was indicated by a constant rate of

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PENICILLIN G

FIG. 1. Structures of benzylsulfonamido penicillin and penicillin G.



BENZYL SULFONAMIDO PENICILLIN

evolution of carbon dioxide until at least two thirds of the substrate was destroyed. Penicillin G was used as substrate in amounts ranging from 1.6 to 40 μm . One unit of enzymic activity is equivalent to the evolution of 1 μm . of carbon dioxide per mg. bacterial protein per hour.* Bacterial protein was calculated from optical densities at 540 m μ of the bacterial suspensions according to a previously determined calibration curve in which bacterial protein was determined by the biuret method with crystalline bovine albumin as standard.

Soluble staphylococcal penicillinase was prepared by disruption of washed staphylococcal suspensions for 20 minutes in the Raytheon 10 kc. sonic oscillator. Cellular debris was removed by centrifugation at approximately 20,000 G. for 30 minutes.

Experiments on induction of penicillinase were performed in the following manner: 48 ml. of a static 16 to 18 hour culture at 37 C. of strain 55-C-1 in tryptic digest broth was diluted with 12 ml. fresh broth and placed in a rotary shaking water bath at 37 C. The pH of the mixture was usually 5.1 to 5.2, but if necessary it was adjusted to this range with *N* hydrochloric acid or *N* sodium hydroxide. For experiments on induction at neutral pH, tryptic digest broth containing 0.05 *M* phosphate buffer pH 7.4 was used for preliminary growth of the organisms and for induction itself. A pH of 7.2 to 7.6 was maintained. Antibiotic solutions in varying concentrations were added at 20 minute intervals beginning at 0 time as follows: 0.125, 0.15, 0.2, 0.25, 0.5, and 0.5 ml. Just before addition of antibiotics, beginning at 20 minutes, the following aliquots were withdrawn and cooled in ice for penicillinase assay: 20, 10, 5, 5, 5, and 5 ml. The values attained at the end of two hours are reported in the tables.

Tube dilution sensitivity tests were performed by adding 1 ml. aliquots of broth dilutions of 24 hour cultures to 1 ml. portions of serial twofold dilutions of antibiotic in tryptic digest broth. After incubation for 24 hours at 37 C. the minimal inhibitory concentration of antibiotic was recorded as the lowest concentration with no visible turbidity.

* Enzymic activity in earlier publications,^{2,3} expressed as μL carbon dioxide/mg. bacterial *N*/hour, may be converted to these units by multiplying by 0.015.

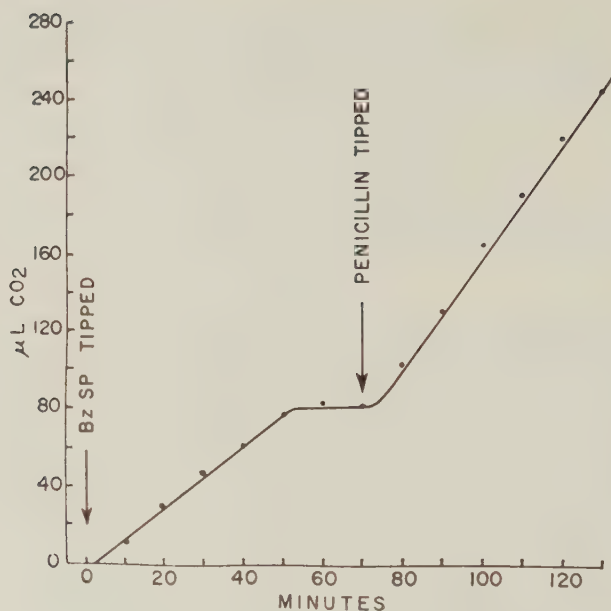


FIG. 2. At 0 minutes, 6-benzylsulfonamidopenicillanic acid ($8.1 \mu\text{m}$) was tipped from one side-arm into a Warburg vessel, which contained as enzyme a suspension of strain 55-C-1. After completion of hydrolysis of benzylsulfonamido penicillin at 50 minutes, $9.4 \mu\text{m}$ of penicillin G were tipped from the second side-arm. Rate of hydrolysis is shown.

RESULTS

Benzylsulfonamido penicillin was hydrolyzed by suspensions of washed staphylococci at a rate $48 (\pm 7)^*$ per cent as rapid as penicillin G (fig. 2). The total carbon dioxide evolved at the completion of the enzymic hydrolysis of benzylsulfonamido penicillin was equivalent to hydrolysis of 40 to 47 per cent of the substrate used. In one experiment, after completion of the enzymic reaction, the contents of the Warburg vessel were passed through a Seitz filter and assayed microbiologically for residual antibiotic activity. None was detected, indicating the inactivation of at least 95 per cent of the initial activity. Benzylsulfonamido penicillin similarly shaken without bacteria in a Warburg vessel and then filtered showed no diminution in antibiotic activity. These results are compatible with the assumption that only one enantiomorph of the racemic synthetic compound is antibiotically active and susceptible to the action of penicillinase. In contrast, with penicillin G, the carbon dioxide evolved is 77 to 93 per cent of the theoretical amount for the quantity of substrate taken, in keeping with the fact that penicillin G, being a single optical isomer, is completely hydrolyzed by the enzyme.

Soluble staphylococcal penicillinase hydrolyzed benzylsulfonamido penicillin $54 (\pm 6)^\dagger$ per cent as rapidly as penicillin G, a rate not appreciably different from that found with suspensions of intact staphylococci. Hence the hydrolysis of benzylsulfonamido penicillin by suspensions of staphylococci is not limited by any barriers restricting access of substrate to the enzyme.

Mixtures of the two penicillins in molar ratios of 0.4 were hydrolyzed by soluble penicillinase at initial rates only slightly lower than that observed with penicillin G alone (table I). Thus there is no evidence of appreciable inhibition of staphylococcal penicillinase by benzylsulfonamido penicillin. These results indicate that the

* Standard deviation of nine experiments.

† Standard deviation of five experiments.

TABLE I

*Hydrolysis of Mixtures of Penicillin G and Benzylsulfonamido
Penicillin by Staphylococcal Penicillinase*

Substrate, μ moles		Penicillinase activity, μ L. carbon dioxide
Penicillin G	Benzylsulfonamido penicillin	
6.5	0	108 \pm 4*
6.5	18	100 \pm 5*

* Mean \pm deviation of duplicate determinations.

affinity of penicillin G for the enzyme is much greater than that of benzylsulfonamido penicillin.⁵

Induction of Penicillinase. We have reported previously that exposure of penicillinase-producing staphylococci to penicillin G under appropriate conditions is followed by an increase in the penicillinase content of the organisms, which is due to inducible (adaptive) synthesis of additional enzyme.² Benzylsulfonamido penicillin is also an inducer of staphylococcal penicillinase, but its effectiveness in this respect relative to that of penicillin G varies with the experimental conditions (table II).

Recent studies have revealed that optimal induction may be achieved by multiple additions of penicillin to broth cultures of staphylococci at pH 5.2.⁷ Under these conditions, additions of penicillin G are followed by marked increases in penicillinase activity per unit organism to values as high as 750 units, in contrast to basal values of 15 to 30 units in the absence of penicillin. Under the same conditions, benzylsulfonamido penicillin is only 1/10 to 1/3 as effective as equivalent concentrations of penicillin G. These experiments were repeated at pH 7.2 to 7.6 in broth buffered with 0.05 M phosphate. At this pH the maximum penicillinase activity attainable by induction with penicillin G is limited to 150 units. Under these conditions and at low inducer concentrations (50 to 200 units/ml.) benzylsulfon-

TABLE II

*Induction of Penicillinase in Staph. aureus 55-C-1 by Penicillin G
and Benzylsulfonamido Penicillin at pH 5.2*

Penicillin G induced		Benzylsulfonamido penicillin induced	
Concentration of inducer, units/ml.	Penicillinase, units	Concentration of inducer, "units"/ml.	Penicillinase, units
2000	765	4000	256
400	428	800	152
80	467	160	46

The concentration of benzylsulfonamido penicillin was twice that of penicillin G in comparable experiments to compensate for the presumably inactive optical isomer in the former. A "unit" of the former is the molecular equivalent of 1 Oxford unit of penicillin G. The initial penicillinase activity prior to induction was 3.4 units.

TABLE III

Induction of Penicillinase in Staph. aureus 55-C-1 by Penicillin G and Benzylsulfonamido Penicillin at pH 7.2 to 7.6

Penicillin G induced		Benzylsulfonamido penicillin induced	
Concentration of inducer, units/ml.	Penicillinase, units	Concentration of inducer, "units"/ml.	Penicillinase, units
400	75	800	70
100	42	200	36
25	31	50	20

Experimental conditions were as in table II except that broth contained 0.05 *M* phosphate buffer, which maintained the pH between 7.2 and 7.6. The initial penicillinase activity prior to induction was 7.0 units.

A "unit" of benzylsulfonamido penicillin is the molecular equivalent of 1 Oxford unit of penicillin G.

amido penicillin was slightly less effective than penicillin G, but at higher concentrations (800 units/ml.) the former was equally effective (table III).

Penicillinase induced by either antibiotic hydrolyzed both penicillins at the same relative rates. To the extent of this test, the enzyme induced by either antibiotic appears to be the same.

Tube Dilution Tests. Serial tube dilution tests were performed with penicillin G, benzylsulfonamido penicillin, and mixtures of the two antibiotics against varying concentrations of staphylococci. The test organism included a strong penicillinase producer strain 55-C-1, a weak penicillinase producer strain 1272, and a penicillin-sensitive strain, strain 9. Results are shown in table IV.

Against the penicillin-sensitive strain, penicillin G is 100 to 200 times as effective an antibiotic as benzylsulfonamido penicillin. As is to be expected with penicillin-sensitive staphylococci, the minimal inhibitory concentration was not influenced by inoculum size. Against penicillinase-producing strains, the superiority of penicillin G is much slighter, particularly in the case of strain 55-C-1, which produces the greater amount of penicillinase. Even at the low inoculum level of

TABLE IV

Relation of Inoculum Size to Minimal Inhibitory Concentration of Penicillin G and Benzylsulfonamido Penicillin

Inoculum	Minimal inhibitory concentration, units/ml.					
	<i>Staph. aureus</i> 55-C-1		<i>Staph. aureus</i> 1272		<i>Staph. aureus</i> 9	
	Penicillin G	Benzylsulfonamido penicillin	Penicillin G	Benzylsulfonamido penicillin	Penicillin G	Benzylsulfonamido penicillin
10 ⁻²	2560	1280	80	80	0.125	20
10 ⁻³	160	160	10	40	0.125	20
10 ⁻⁴	40	40	2.5	40	0.125	20
10 ⁻⁵	10	40	1.25	40	0.125	20

Inoculum was the indicated dilution of an overnight culture.

A "unit" of benzylsulfonamido penicillin is the molecular equivalent of 1 Oxford unit of penicillin G.

TABLE V

Inhibition Test with Mixtures of Penicillin G and Benzylsulfonamido Penicillin Against a Weak Penicillinase-producing Staphylococcus

Penicillin G, units/ml.	Benzylsulfonamido penicillin, "units"/ml.				
	0	4.5	9	18	36
0	+	+	+	+	—
5	+	+	+	+	—
15	+	+	+	±	—
45	+	+	—	±	—
135	—	—	—	—	—

Each tube contained 0.5 ml. of dilutions of each of the antibiotics and 0.5 ml. of a 10^{-2} dilution of an overnight culture of *Staph. aureus* strain 6549. Results were read by inspection after 24 hours' incubation at 37 C.

A "unit" of benzylsulfonamido penicillin is the molecular equivalent of 1 Oxford unit of penicillin G.

a 10^{-5} dilution of an overnight culture, penicillin G is only four times as effective as benzylsulfonamido penicillin, and only two times as effective if one excludes as inactive one of the optical isomers of the synthetic compound. As the inoculum increases, the minimal inhibitory concentration of penicillin G increases more rapidly than that of benzylsulfonamido penicillin, with the result that with inoculums a 10^{-2} dilution, penicillin G is less effective than benzylsulfonamido penicillin.

The possibility was considered that some of the difference in results between the two penicillins might be due to the fact that benzylsulfonamido penicillin was tested as the N-ethylpiperidine salt while penicillin G was the sodium salt. However, tube dilution sensitivity tests with mixtures of sodium penicillin G and equivalent amounts of N-ethylpiperidine gave the same results as those with penicillin G alone.

Tube dilution tests with mixtures of benzylsulfonamido penicillin and penicillin G in varying proportion gave evidence of a slight additive effect but no true synergism (table V).

DISCUSSION

The foregoing observations indicate that benzylsulfonamido penicillin is a relatively weak antibiotic hydrolyzed by staphylococcal penicillinase one half as rapidly as penicillin G. A direct consequence of this relative resistance to penicillinase is the increasing efficiency of benzylsulfonamido penicillin relative to penicillin G with increasing size of inoculum of a penicillinase-producing *Staphylococcus* in the tube dilution test. Increase in inoculum size necessarily signifies a proportional increase in the penicillinase content of the inoculum. In consequence, the initial penicillin concentration must be increased if an inhibitory concentration is to be maintained for the time necessary to prevent visible growth at 24 hours. For any increment in inoculum, only one half as much benzylsulfonamido penicillin as penicillin G will be required since the additional penicillinase hydrolyzes only one half as much of the former as of penicillin G. Since the incremental requirement of penicillin G will increase twice as rapidly as that of benzylsulfonamido penicillin, the minimal inhibitory concentration for penicillin G must eventually surpass that for benzylsulfonamido penicillin, as in fact it does with a 10^{-2} dilution of strain 55-C-1.

Induction of increased amounts of penicillinase by either of the two penicillins may contribute to an increase in minimal inhibitory concentration of antibiotic. From our results it seems probable that in a neutral environment both penicillins are almost equally effective in this respect, but that in a more acidic environment, as may be found in inflammatory foci,⁶ the very high levels of penicillinase induced by penicillin G may contribute to reducing the antibiotic effectiveness of penicillin G relative to benzylsulfonamido penicillin.

In its relative resistance to staphylococcal penicillinase, benzylsulfonamido penicillin resembles other penicillins that differ from penicillin G in the chemistry of their side chains. These include cephalosporin N,⁵ penicillin K,⁴ and penicillin F,⁴ which have been reported to be hydrolyzed by *Bacillus cereus* penicillinase more slowly than penicillin G. Penicillin V, on the other hand, is hydrolyzed by the same enzyme 1.25 times as rapidly as penicillin G. Inhibition by antibiotics of the enzymic activity of penicillinase has been reported only with cephalosporin C,⁵ an antibiotic related to penicillin G but differing in its ring structure as well as the side chain. Since the ring structure of benzylsulfonamido penicillin is the same as that of penicillin G, it is not surprising that the former does not inhibit the action of penicillinase and, in consequence, does not synergize with penicillin G.

In view of its low intrinsic activity, benzylsulfonamido penicillin will probably be of no practical value in treatment of penicillin-resistant staphylococcal infections. The small amount of material available to us has precluded chemotherapeutic trials. Nevertheless, further search for synthetic penicillins may be rewarding, since even with relatively weak intrinsic antibiotic activity, a synthetic penicillin might be useful in therapy if it were highly resistant to staphylococcal penicillinase and could be administered safely in the large dosages feasible with penicillin G.

SUMMARY

Benzylsulfonamido penicillin, a synthetic analogue of penicillin G, is hydrolyzed by staphylococcal penicillinase about one half as rapidly as penicillin G. At pH 5.2, benzylsulfonamido penicillin is one third to one tenth as effective an inducer of staphylococcal penicillinase as is penicillin G. At neutral pH, benzylsulfonamido penicillin is nearly as effective as penicillin G.

The minimal inhibitory concentration of benzylsulfonamido penicillin for a penicillin-sensitive *Staphylococcus* is 100 to 200 times that of penicillin G. With increasing size of inoculum of a penicillinase-producing *Staphylococcus*, the difference in minimal inhibitory concentration of these agents diminishes. With the largest inocula used, the minimal inhibitory concentration for benzylsulfonamido penicillin is less than that for penicillin G. This relation is a logical consequence of the differences in susceptibility to penicillinase of the two antibiotics.

ACKNOWLEDGMENTS

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The Effect of Ascorbic Acid on the Absorption of Potassium Phenoxymethyl Penicillin (Penicillin V)

M. HEROLD, M. HEŘMANSKÝ, O. ŠMAHEL, AND V. VLČEK

*Antibiotic Research Institute, Rožtoky, and Clinic for Internal Medicine,
Postgraduate Medical School, Praha, Czechoslovakia*

The present study is a continuation of a systematic search for substances that could enhance the absorption in the organism of orally administered penicillin. Originally begun with penicillin G,¹ this study was later, after the introduction of penicillin V,^{2,3} extended to include the potassium salt and the free acid form of this biosynthetic penicillin. A number of substances investigated in the course of this study (phenacetin, caffeine, quinine, acetylsalicylic acid) was found to lack any adjuvant action, and in the case of aminopyrine, which had been demonstrated to prolong and slightly enhance blood levels of penicillin G,^{1,4} this effect was lost in combination with penicillin V.⁵ The effect of stearic acid was confirmed with both procaine penicillin G and penicillin V and the inclusion of 1 per cent of this acid was adopted as a standard constituent of tablet formulations.^{4,5}

Some unexpected results of experiments with citric and ascorbic acid will be described.

MATERIALS AND METHODS

Tablets containing 200,000 units of either penicillin G (as procaine penicillin G) or penicillin V (equivalent to 125 mg. of penicillin V acid, as the free acid or potassium salt) were administered together with the studied adjuvant, which was usually contained in a starch capsule. Citric acid was used in single doses of 200 and 400 mg., ascorbic acid in doses of 100, 200, and 400 mg. In the first stage of the study, groups of 5 patients chosen at random in the wards of the Clinic were used for each drug combination. All of the patients used in this study were within the age group between 20 and 60 years. After this preliminary screening, the combinations that seemed to give significantly higher plasma levels than the penicillin preparation alone, were submitted to cross experiments, using a larger group of subjects. In the present case, the combination of 200,000 units of potassium penicillin V with 400 mg. of ascorbic acid was given to 20 healthy volunteers from 20 to 35 years old; each of these volunteers received also (on a different day) a potassium penicillin V tablet (200,000 u.) without ascorbic acid.

Serum concentrations of penicillin were determined in blood samples, taken 1, 3, 4 (in some instances 5), 6, and (sometimes) 8 hours after the administration, by a modified serial dilution method, using *Staphylococcus aureus* MAU as test organism.⁶ In the cross experiment, only samples at 1, 3, and 6 hours were taken.

In all instances, subjects were given the tablet and the adjuvant to swallow 30 minutes before breakfast. All tablets in each experiment were of an identical batch,

TABLE I
Penicillin Concentrations in Human Plasma

Penicillin preparation	No. patients	Units per ml. of serum, hours				
		1	3	4	6	8
Procaine penicillin G tablets	5					
Range		0.12-0.96	0-0.24	—	0-0.18	0-0.12
Average		0.65	0.16	—	0.07	0.05
Positive levels		5/5	4/5		3/5	3/5
Procaine penicillin G tablets plus citric acid (200 mg.)	5					
Range		0.18-0.36	0.09-0.24	—	0-0.06	0
Average		0.25	0.13	—	0.01	0
Positive levels		5/5	4/5		1/5	0/5
Procaine penicillin G tablets plus citric acid (400 mg.)	5					
Range		0.36-0.72	0.06-0.24	—	0-0.06	0
Average		0.48	0.18	—	0.03	0
Positive levels		5/5	5/5	—	3/5	0
Procaine penicillin G tablets plus ascorbic acid (100 mg.)	5					
Range		0.24-0.96	0.06-0.36	—	0-0.12	0-0.03
Average		0.58	0.18	—	0.06	0.006
Positive levels		5/5	5/5	—	4/5	1/5
Procaine penicillin G tablets plus ascorbic acid (200 mg.)	5					
Range		0.12-0.96	0.18-0.48	—	0.03-0.12	0-0.03
Average		0.62	0.26	—	0.08	0.01
Positive levels		5/5	5/5	—	5/5	2/5
Procaine penicillin G tablets plus ascorbic acid (400 mg.)	5					
Range		0.36-0.96	0.12-0.24	—	0.03-0.24	0-0.03
Average		0.65	0.22	—	0.12	0.02
Positive levels		5/5	5/5	—	5/5	3/5

in order to eliminate differences in disintegration time and crystal size of the active substances.

RESULTS

In tables I, II, and III serum concentrations in patients following the administration of the investigated combinations are recorded. The mean values of serum levels are plotted in figures 1, 2, and 3. Although experiments with such small groups can only give a rough estimate of the combination's effectiveness, a more or less general pattern may be seen in the action of citric acid, which diminishes the initial level in practically all cases.

The effect of ascorbic acid is very interesting, in that it is practically nil with procaine penicillin G and the free acid of penicillin V (tables I and II, figs. 1 and 2), but it is indisputably evident with the potassium salt of penicillin V. The effect, according to table III and figure 3, is definitely dependent upon the dose of ascorbic

TABLE II

Penicillin Concentrations in Human Plasma

Penicillin preparation	No. patients	Units per ml. of serum, hours				
		1	3	4	6	8
Penicillin V, free acid	20					
Range		0.24-3.84	0.03-0.32	0.02-0.48	0-0.12	0-0.03
Average		1.65	0.16	0.13	0.02	0.003
Positive levels		20/20	10/10	10/10	6/20	1/10
Penicillin V, free acid, plus citric acid (200 mg.)	5					
Range		0.08-1.44	0.12-1.28	0-0.64	0-0.08	—
Average		0.82	0.47	0.19	0.02	—
Positive levels		5/5	5/5	4/5	2/5	—
Penicillin V, free acid, plus citric acid (400 mg.)	5					
Range		0.36-0.96	0.12-0.36	0.03-0.12	0-0.03	—
Average		0.79	0.22	0.09	0.01	—
Positive levels		5/5	5/5	5/5	2/5	—
Penicillin V, free acid, plus ascorbic acid (100 mg.)	5					
Range		0.96-2.88	0.12-0.72	0.12-0.48	0-0.09	—
Average		1.82	0.31	0.23	0.02	—
Positive levels		5/5	5/5	5/5	2/5	—
Penicillin V, free acid, plus ascorbic acid (200 mg.)	5					
Range		0.96-2.56	0.16-0.48	0.008-0.24	0-0.02	—
Average		1.73	0.34	0.14	0.01	—
Positive levels		5/5	5/5	5/5	3/5	—
Penicillin V, free acid, plus ascorbic acid (400 mg.)	5					
Range		1.28-2.56	0.08-0.64	0.16-0.64	0-0.24	0
Average		1.79	0.4	0.37	0.08	0
Positive levels		5/5	5/5	5/5	3/5	0/5

acid used, the 100 mg. level being scarcely effective. To confirm the effect of ascorbic acid at the 400 mg. level, not only was the group of patients augmented to 10, but the cross experiment (table IV, fig. 4) was run, using 20 volunteers. While in the patient group the penicillin level was practically trebled by the administration of ascorbic acid, the cross experiment substantiated this merely to the extent of the level being doubled, yet nearer examination of the levels in individual subjects in table IV shows the enhancing effect to be remarkably consistent. It should be also noted that the difference in the experiments with patients and volunteers partly lies in the average concentrations following the administration of potassium penicillin V alone, which is definitely lower in the patient group, as confirmed also using tablets of a different batch. Besides increasing the initial levels, ascorbic acid seems to prolong the plasma levels beyond the sixth hour as well—in the cross experiment 45 per cent of the subjects had measurable levels of penicillin in this hour, compared to only 5 per cent in the controls.

TABLE III

Penicillin Concentrations in Human Plasma

Penicillin preparation	No. patients	Units per ml. of serum, hours			
		1	3	4	6
Potassium penicillin V (batch A) tablets	20				
Range		0.96-1.92	0.04-0.32	0.03-0.12	0.0-0.04
Average		1.38	0.18	0.08	0.02
Positive levels		20/20	20/20	10/11	1/14
Potassium penicillin V (batch B) tablets	5				
Range		0.96-1.92	0.06-0.24	0-0.06	0
Average		1.44	0.16	0.03	0
Positive levels		5/5	5/5	4/5	0/5
Potassium penicillin V (batch B) plus citric acid (200 mg.)	5				
Range		0.64-0.96	0.06-0.32	0-0.06	0
Average		0.77	0.16	0.02	0
Positive levels		5/5	5/5	4/5	0/5
Potassium penicillin V (batch B) plus citric acid (400 mg.)	5				
Range		0.48-1.44	0.03-0.36	0-0.03	0
Average		0.72	0.12	0.02	0
Positive levels		5/5	5/5	3/5	0/5
Potassium penicillin V (batch B) plus ascorbic acid (100 mg.)	5				
Range		0.96-2.56	0.16-0.32	0.16-0.32	0-0.16
Average		1.47	0.23	0.19	0.09
Positive levels		5/5	5/5	5/5	3/5
Potassium penicillin V (batch B) plus ascorbic acid (200 mg.)	5				
Range		1.44-3.84	0.72-1.92	0-0.24	0-0.03
Average		2.72	1.49	0.1	0.01
Positive levels		5/5	5/5	4/5	2/5
Potassium penicillin V (batch B) plus ascorbic acid (400 mg.)	10				
Range		2.56-5.76	0.16-1.44	0.12-0.96	0-0.12
Average		3.97	0.95	0.29	0.05
Positive levels		10/10	10/10	10/10	7/10

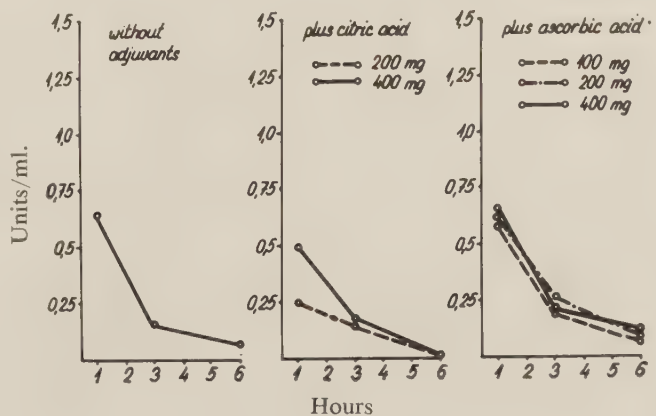


FIG. 1. Serum concentrations of penicillin following the oral administration of 200,000 units of procaine penicillin G with and without citric and ascorbic acid.

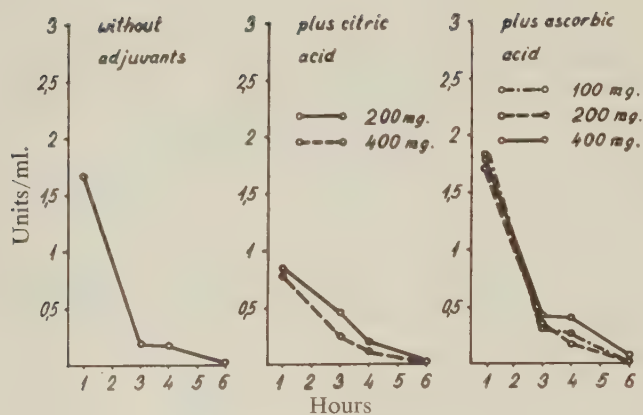


FIG. 2. Serum concentrations of penicillin following the oral administration of 200,000 units of the free acid of penicillin V with and without citric and ascorbic acid.

DISCUSSION

We are aware that these results ought to be supplemented with determinations of serum concentrations following the administration of the preparations after a meal, and that the omission of taking blood samples at one half hour or at two hours has perhaps in some instances cut off the true peak of the absorption curves. These additional data unfortunately could not be gathered in time for this communication and will be published later. Even without them, however, it seems clear that ascorbic acid does possess a definite adjuvant action when administered together with penicillin V. Why this is so only with the potassium salt of this penicillin and not with the free acid is rather puzzling. The effect of citric acid, not very illuminative in itself, has been included in this communication because it seems to exclude certain explanations of the effect of ascorbic acid, namely, those based on a buffering or sequestering action; the differences between the action of probenecid (which is an active adjuvant of the free acid of penicillin V^{7, 8}), aminopyrine (ineffective with penicillin V⁵), and ascorbic acid seem to point to a new adjuvant mechanism with the latter substance, which warrants further investigation.

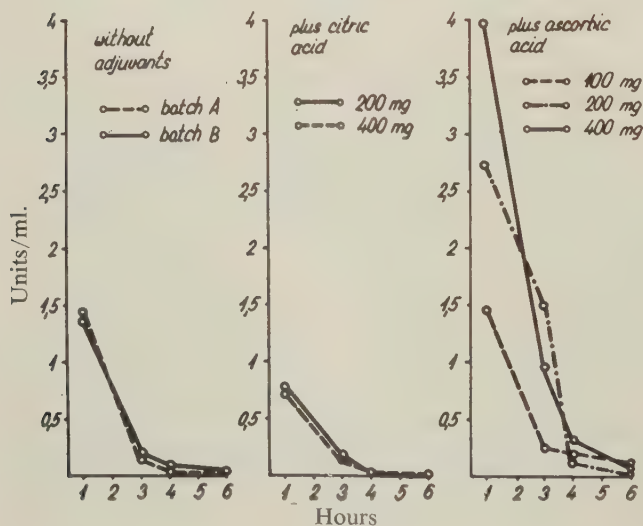


FIG. 3. Serum concentrations of penicillin following the oral administration of 200,000 units of potassium penicillin V with and without citric and ascorbic acid.

TABLE IV

Results of a Cross Experiment on 20 Healthy Volunteers: 200,000 Units of Potassium Penicillin V Tablets (Batch B) with and without Ascorbic Acid (400 mg.)

Subject	Units per ml. of serum, hours					
	1		3		6	
	Without ascorbic acid	With ascorbic acid	Without ascorbic acid	With ascorbic acid	Without ascorbic acid	With ascorbic acid
J.D.	0.64	1.92	0.32	1.92	0.04	0.03
E.F.	1.28	2.88	0.64	0.96	0.04	0.03
J.D.	1.92	3.84	0.32	1.44	0.00	0.00
A.N.	1.92	3.84	0.64	0.24	0.00	0.03
J.N.	1.28	1.92	0.32	1.44	0.00	0.03
Z.S.	1.92	1.28	0.64	1.92	0.00	0.03
D.S.	0.96	3.84	0.64	0.24	0.00	0.00
V.V.	1.92	1.92	0.48	0.72	0.00	0.06
A.F.	1.28	3.84	0.64	0.24	0.00	0.00
E.V.	1.92	3.84	0.24	0.09	0.00	0.00
L.B.	0.96	1.92	0.24	1.44	0.00	0.03
Z.C.	2.88	2.88	0.48	0.96	0.00	0.00
B.H.	1.92	3.84	0.48	1.44	0.00	0.00
A.L.	0.96	1.92	0.12	0.48	0.00	0.00
M.M.	1.44	1.44	0.48	0.48	0.00	0.00
B.S.	1.44	2.88	0.36	1.44	0.00	0.03
M.S.	1.92	2.88	0.48	0.96	0.00	0.03
H.S.	0.96	2.88	0.24	0.24	0.00	0.00
L.B.	0.96	3.84	0.18	0.48	0.00	0.00
H.V.	1.44	3.84	0.12	0.24	0.00	0.00
Av.	1.54	2.87	0.40	0.86	0.00	0.015

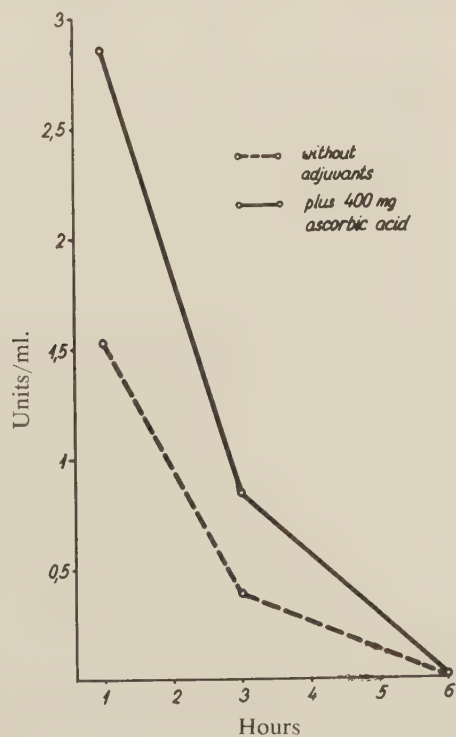


FIG. 4. Average penicillin serum concentrations in 20 volunteers after receiving 200,000 units potassium penicillin V either alone or with 400 mg. of ascorbic acid.

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A Comparative Study of Inhibition of Penicillinase by Simple Compounds, Antiserum, and Their Combinations

EDWARD A. CARR, JR.

*Departments of Pharmacology and Internal Medicine,
University of Michigan, Ann Arbor, Michigan*

Most penicillin-resistant strains of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) isolated from human infections produce penicillinase. As penicillin's relatively low toxicity, inexpensiveness, and rapid bactericidal action make it an outstandingly useful drug in the treatment of infections caused by strains of *M. pyogenes* var. *aureus* that are still sensitive to it, the possibility of inhibiting penicillinase in the hope of abolishing resistance of many strains of this organism has attracted considerable attention. Housewright and Henry¹ and Perlstein and Liebmann^{2,3} showed that antiserum against penicillinase could be prepared and that such antiserum neutralized in vitro the effect of penicillinase on penicillin. Markov et al⁴ indicated that they had found such antiserum to be effective also in increasing the effect of penicillin on previously resistant micrococci. Unfortunately, Housewright and Henry¹ noted only a slight effect of antipenicillinase serum in increasing bacterial sensitivity to penicillin, and the experiments of Pollock⁵ suggested plausible reasons for this failure. Penicillinase is apparently both extracellular and intracellular. The inaccessibility of the latter penicillinase to the large antibody molecule is at least one probable reason for this failure.

METHODS

Six rabbits, each weighing approximately 2.5 Kg., were immunized by a modification of the method of Fisher et al,⁶ employing Freund's adjuvant. Each injection of penicillinase (Neutrapen*) consisted of 230,000 units in 0.4 ml. of a vehicle containing, in addition to killed mycobacteria, 7.5 per cent mannide mono-oleate (Arlacel A†), 42.5 per cent light paraffin oil (Bayol F‡), and 50 per cent isotonic sodium chloride solution, by volume. Each course consisted of five injections given over a two week period. Skin and precipitin tests, with appropriate controls, were performed during the third or fourth week after conclusion of a course. Antiserum was subsequently harvested by bleeding the rabbits under urethane anesthesia. After preliminary storage at approximately 0 C. for a short period of time, the antisera were kept at approximately -40 C. until ready for use. The two best antisera were chosen for the experiments to be described. Antiserum A was obtained from a rabbit that had received a course of intracutaneous injections followed by a course of intramuscular injections, and antiserum B was obtained from a rabbit that had received the two courses of injections in reverse order. The courses were separated by an interval of six weeks. Human blood was obtained by venipuncture,

* The trade name of SchenLabs Pharmaceuticals for penicillinase is Neutrapen.

† The trade name of Atlas Powder Co. for mannide mono-oleate is Arlacel A.

‡ The trade name of Penola Oil Co. for light paraffin oil is Bayol F.

and the serum was used in the same manner as rabbit serum. Control rabbit serum was obtained from rabbits that had received no injections for immunization or skin testing and control human serum was obtained from uninfected persons.

The effect of penicillinase was measured by a modification of the iodometric method of Perret.⁷ The final volume always consisted of either 8 or 9 ml., all flasks in a given run containing the same volume. In some of the experiments involving antiserum alone, 5 ml. of 0.007 *M* potassium benzyl penicillin was added, as in the original method, but in all other experiments 5 ml. of 0.0021 *M* potassium benzyl penicillin was added. The 2 ml. of enzyme solution contained 0 to 400 units of penicillinase, the majority of experiments employing three concentrations: 0, 40, and 120 units. The additional 1 or 2 ml. in each flask consisted of antiserum, other inhibitor solution, additional buffer, or various combinations of these. Each run contained controls for spontaneous breakdown of penicillin and controls for interference of the inhibitor solution or antiserum with the iodine titration. Inhibitors other than antiserum were introduced into the reaction mixture as 1 ml. of a solution of the inhibitor in buffer or in certain other solvents. The other solvents* were 0.001 sodium hydroxide, propylene glycol, 10 per cent dimethylformamide, 10 or 50 per cent ethylene glycol, 10 per cent ethanol, or 10 per cent dipropylene glycol methyl ether (Dowanol 50 B†). These solvents were chosen because experiments showed that they themselves caused ≤ 5 per cent inhibition of penicillinase in the concentrations used. When such solvents were used, additional appropriate controls were included in the run.

The molarity given for the various inhibitors is the molarity in the final reaction mixture. Where breakdown of penicillin is expressed as units inactivated per given time, control values have already been subtracted. Inhibition was calculated as follows:

$$I = 100 - 100 \left(\frac{U_{ip} - U_1}{U_p - U_o} \right)$$

where I = inhibition (expressed as per cent);

U_{ip} = units of penicillin inactivated in presence of inhibitor and penicillinase,

U_1 = units of penicillin inactivated in presence of inhibitor alone,

U_p = units of penicillin inactivated in presence of penicillinase without inhibitor,

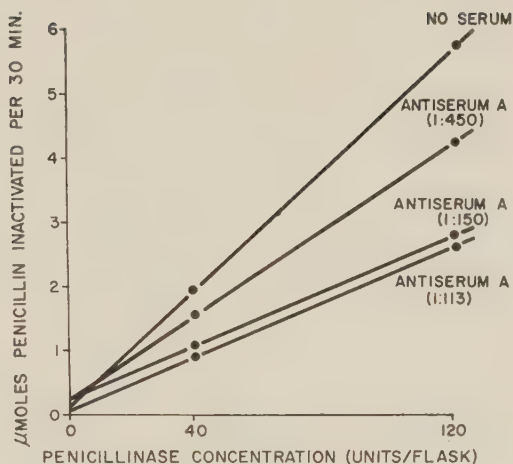
U_o = units of penicillin inactivated in absence of both penicillinase and inhibitor.

In experiments in which antiserum and another inhibitor were used in combination, it was necessary to know the degree of inhibition expected from the same concentration of each one singly. Separate runs established curves for the relation

* The concentration of each of these solvents refers to the added solution, not the final concentration in the flask. The percentages are expressed by volume, and the additional solvent in the added solution was buffer.

† The trade name of Dow Chemical Co. for dipropylene glycol methyl ether is Dowanol 50 B.

FIG. 1. Inhibition of penicillinase by antiserum of rabbit A.

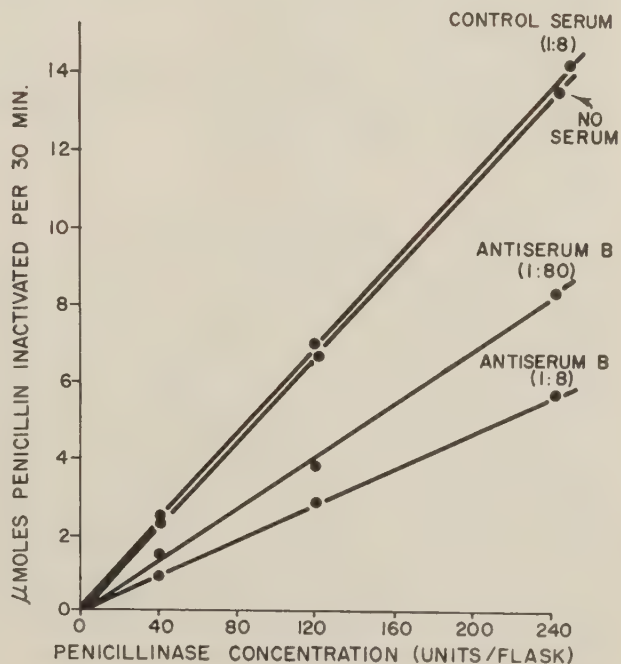


between concentration and inhibition with antiserum, benzoate, salicylate, and so on. However, in any run in which combinations of antiserum and other inhibitors were used, we ensured against the possibility of significant variation in the runs from day to day by confirming each time at least one point on the standard curve for antiserum alone and one point on the standard curve for the other inhibitor alone. This was carried out by the inclusion of a flask containing antiserum without other inhibitor and one containing the other inhibitor without antiserum.

RESULTS

None of the following compounds, tested at a concentration of $10^{-3} M$, gave

FIG. 2. Inhibition of penicillinase by antiserum of rabbit B.



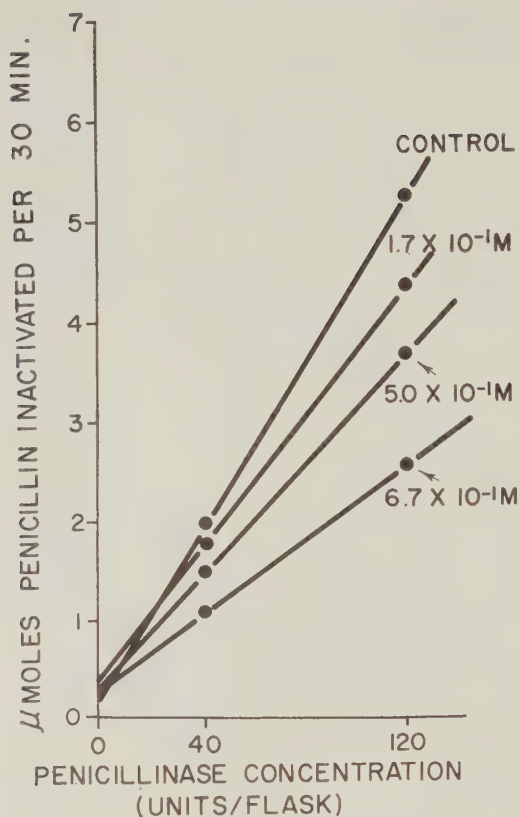


FIG. 3. Inhibition of penicillinase by benzoate.

≥ 10 per cent inhibition of *Bacillus cereus* penicillinase: sodium salicylate, sodium benzoate, *p*-aminosalicylic acid, potassium *p*-aminobenzoate, methyl propyl ketone, sorbic acid, chiniofon, chloroquine, carbutamide, thiouracil, 2-methyl-2-mercapto-propane, 3-quinoline carboxylic acid, 8-amino-5-chloroquinoline, 7-methoxy-1-methylquinolinium iodide, *p*-chlorobenzenesulfonamide, and *p*-bromobenzenesulfonamide. Phenethylbiguanide gave 11 per cent inhibition, while the results with chlorpropamide showed unexplained variability but did not suggest potent inhibition.

None of the following compounds gave ≥ 10 per cent inhibition when tested at a concentration of 10^{-4} M: 2, 3-dichloroquinoxaline, 4-chlorobenzenesulfonanilide, iodosobenzene, *p*-chlorobenzenesulfonic acid hydrazide, chlorophenesin, diphenyliodonium iodide, 4, 7-dichloroquinoline, 4'-chlorobenzenesulfonanilide, 2, 8-dichloro-10 (3-dimethylaminopropyl) phenothiazine hydrochloride, 4-chloro-*o*-phenylene diamine, meclizine hydrochloride, 3-chloro-10 (3-dimethylaminopropyl) phenothiazine hydrochloride, and 2', 3'-dichlorobenzenesulfonanilide. Iodochlorhydroxyquin showed no significant inhibition at 10^{-5} M. Compounds tested at concentrations weaker than 10^{-3} M were those showing definite or questionable precipitation in the reaction mixture at 10^{-3} M. All reaction mixtures were carefully examined for clarity and no experiment was considered valid if there was any question of imperfectly dissolved inhibitor, a source of error noted by previous workers.⁸

Inhibition of penicillinase by various concentrations of antiserum from rabbits A and B, benzoate, salicylate, and *p*-aminobenzoate is shown in figures 1, 2, 3, and 4.

Inhibition of penicillinase by combinations of antiserum and benzoate, salicylate, or *p*-aminobenzoate is shown in table I. Combining the inhibitors markedly decreased their effect.

None of the following patients' sera significantly inhibited *B. cereus* penicillinase: 3 patients with infections caused by penicillin-resistant *M. pyogenes* var. *aureus*, 1 patient with an infection caused by a penicillin-sensitive strain of the same organism, and 2 patients without histories of recent infection by this organism. The infections included genitourinary and skin infections, and at least two of the penicillin-resistant infections were recorded as having been present for at least two weeks.

DISCUSSION

Except for the experiments with human sera, where failure to demonstrate inhibition simply indicated the absence of antibodies to staphylococcal penicillinase capable of giving a cross reaction with *B. cereus* penicillinase, all other experiments reported employed *B. cereus* penicillinase and antibodies against it. Therefore, internal consistency of the method makes this a reasonable model for penicillinase inhibition. However, it seems more reasonable to apply the principles rather than the details derived from this model to penicillinase from other species. In particular, it

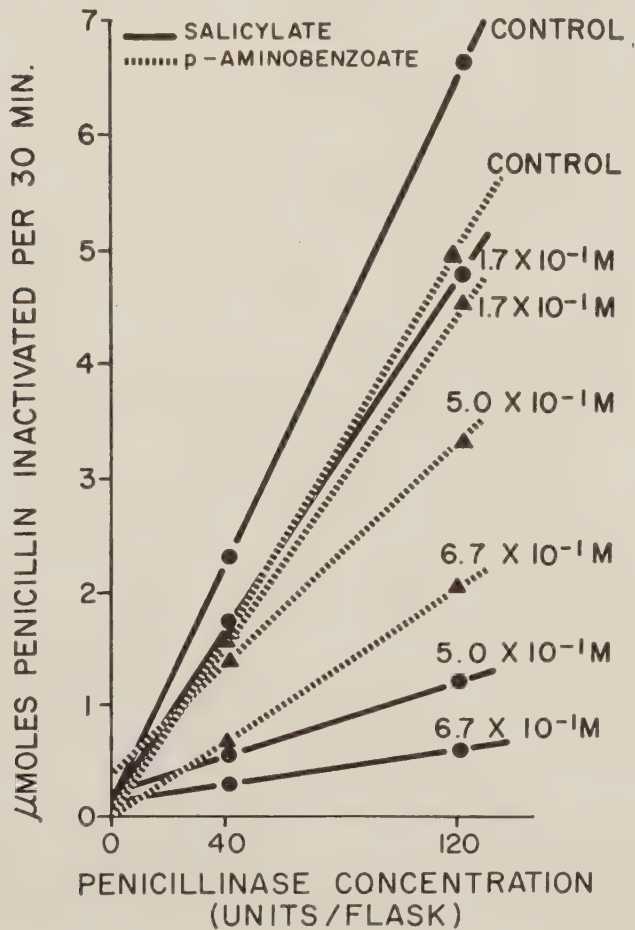


FIG. 4. Inhibition of penicillinase by salicylate and *p*-aminobenzoate.

TABLE I

*Inhibition of Penicillinase in Vitro by Combinations of
Rabbit Antiserum and Benzoate or Its Derivatives*

Serum (concentrations 1:113 to 1:450)	Other inhibitor (concentrations $1.7 \times 10^{-1}M$ to $6.7 \times 10^{-1}M$)	Inhibition (%) expected from serum alone in concentration used	Inhibition (%) expected from other inhibitor alone in con- centration used	Inhibition (%) observed with combination
Antiserum A	Benzoate	21	25	13
Antiserum A	Benzoate	25	31	16
Antiserum A	Benzoate	36	17	12
Antiserum A	Benzoate	40	22	20
Antiserum A	Benzoate	47	11	22
Antiserum A	Benzoate	51	17	29
Antiserum B	Benzoate	40	25	47
Antiserum B	Benzoate	43	22	29
Control	Benzoate	0	22	22
Control	Benzoate	<1	17	27
Control	Benzoate	<6	17	27
Antiserum A	Salicylate	36	54	28
Antiserum A	Salicylate	40	61	35
Antiserum A	Salicylate	41	57	22
Antiserum A	Salicylate	50	53	5
Control	Salicylate	-1	58	57
Control	Salicylate	0	55	58
Control	Salicylate	1	50	52
Control	Salicylate	2	56	56
Antiserum A	<i>p</i> -Aminobenzoate	36	9	-30
Antiserum A	<i>p</i> -Aminobenzoate	40	16	9

cannot be assumed that a compound showing a certain degree of inhibition in our experimental model will show the same degree of inhibition in other types of experiment. Our results confirm the previous report⁹ that benzoate is a definite but fairly weak inhibitor, while chloroquine did not show in our experiments the inhibitory power clearly found by other workers¹⁰ who used staphylococcal penicillinase.

The results shown in figures 1, 2, 3, and 4 strongly suggest that rabbit antiserum, benzoate, and the two benzoate derivatives studied inhibit penicillinase in a reversible manner. Ackermann and Potter¹¹ showed that curves similar to those presented here are typical of reversible inhibition, whereas curves for irreversible inhibitors cross the X-axis to the right of the origin by an amount that is proportional to the amount of inhibitor.

The effect of a reasonably potent antiserum on a relatively weak inhibitor such as benzoate was of particular interest, because the possible potentiation of a relatively weak inhibitor of small molecular weight by antiserum would appear to be one possible practical use for such antiserum. Although conclusions from our experimental system cannot, as noted previously, be taken as strictly applicable to human infections with penicillin-resistant organisms, the failure of the present experiments to reveal potentiation offers in principle still further discouragement for the practical use of antiserum, even in combination with other inhibitors, in penicillin-resistant infections. However, all the present experiments combining antiserum with another inhibitor employed salicylate or a related compound as the other inhibitor. As salicylate has been previously reported to interfere with certain antigen-antibody reac-

tions, it is not permissible to conclude that the results observed here would necessarily apply to *all* penicillinase inhibitors of low molecular weight.

The antagonism observed may be of more fundamental interest, however. As the relation between inhibition and concentration of an inhibitor may not always be linear when plotted on an arithmetic scale, it would not be expected that arithmetic addition of the inhibition obtained with each of two inhibitors separately would necessarily equal the inhibition caused by a combination of the two, even if the two, from the pharmacological standpoint, acted synergistically. However, the present experiments showed that the effect of a combination of antiserum plus other inhibitor was in 13 of 14 experiments less than the effect of the more potent of the two used singly, and in 10 of the 14 experiments less than the effect of either of the two used singly.

The antagonism observed may be specific, i.e., those active groups on each inhibitor molecule that are responsible for inhibition of penicillinase may be the same groups that take part in the reaction between the inhibitors. However, one must also consider the possibility of nonspecific antagonism, in which portions of the molecules not concerned with penicillinase inhibition take part in the reaction between inhibitors. The binding of simple chemical compounds by serum proteins is well known. Conversely, the relatively high concentration of benzoate and its derivatives used here suggests the possibility that they may have caused nonspecific degradation of the antibody protein, despite the absence of any visible evidence of precipitation of the protein. However, a comparison of the results obtained with antiserum and those obtained with control sera, as shown in table I, suggests specific antagonism. Three different control sera, including one obtained by exsanguinating a normal rabbit under urethane anesthesia, as in the case of the immunized rabbits, were individually used in the experiments reported in table I. All these sera failed to show the antagonism noted with antiserum.

The reaction between the small molecule, penicillin, and the large molecule, penicillinase, may thus be inhibited either by a small molecule, such as benzoate or some of its derivatives, or a large antibody molecule. In the experimental design used here, the addition of both types of inhibitors simultaneously appeared to result in a reaction between one inhibitor and the other rather than a reaction with penicillinase.

SUMMARY

1. Rabbit antiserum, benzoate, salicylate, and *p*-aminobenzoate each inhibited penicillinase when used singly in vitro, as shown by the iodometric method.
2. The combination of antiserum and benzoate or benzoate derivative resulted in antagonism rather than synergism between the inhibitors.

ACKNOWLEDGMENTS

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Strauss of SchenLabs Pharmaceuticals, Inc., New York City. Many of the simple chemical compounds studied were supplied by Dr. Joseph P. Webb of the Upjohn Co., Kalamazoo, Mich. Killed mycobacteria for the Freund's adjuvant were supplied by Dr. A. H. Wheeler of the University of Michigan. The interest and support of these is acknowledged with appreciation.

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Inhibition of Penicillinase and Reduction of Resistance in Vitro in Staphylococci by Surface-Active Substances

R. BRUNNER, A. KRAUSHAAR, AND E. PROHASKA

Research Department of the Biochemie G.m.b.H., Kundl (Tyrol), Austria

Previous investigations¹ showed that, with regard to optimal inhibition of their penicillinase, highly resistant strains of staphylococci behave in vitro like normally sensitive strains with respect to penicillin. In view of the fact that substances inhibiting penicillinase in these experiments were surface-active substances of a mainly anion-active nature, this paper is intended to show to what extent a relationship may exist between effective intensity and surface activity. The latter is determined by measuring the surface tension of the aqueous solution of these substances. The more the surface tension of distilled water (73.4 dynes/cm. at 20 C.) is reduced, the greater will be the surface activity.

Our investigations were carried out with commercial products that were partly purified by means of a special method:¹ Alcylsulfates: sodium tridecyl-2-sulfate (cleaning method), sodium laurylsulfate, purity U.S.P., cocinic acid-ethanolamide-sulfate; alcylsulfonates: Mersolat H (cleaning method), Igepon AP (cleaning method), Rapidnetzer BASF (detergent) (cleaning method), Igepon T 30 per cent; alcyl-aryl-sulfonates: sodium-dodecylbenzenesulfonate (cleaning method), Leonil DB, Ultravon K, Rapidnetzer RBD (detergent), Lissapol LS, Nekal BX; and sodium apocholate and sodium dehydrocholate. In the concentrations used for our tests these substances themselves showed no inhibition in the tube test to our test strains.

METHODS

Measurement of Surface Tension. Surface tension was measured according to the principle of the duNouy tensiometer by means of torsion scales according to the loop method, a measuring wire of definite length (L) and diameter (2 r) being used. Surface tension was determined in mg./ml. according to the equation:

$$a = \frac{P'' - P'}{2L} - r \cdot \sqrt{2 \cdot a' \cdot s} + 0.785 \cdot r^2 \cdot s + \frac{2 \cdot r \cdot a'}{L}$$

where P' = weight of the immersed loop when empty,

P'' = breaking resistance of the loop,

$$a' = \frac{P'' - P'}{2L},$$

s = specific weight of the liquid.

The surface tension in dynes/cm. is obtained by multiplication with the factor 9.81.

Testing Penicillinase Activity by the Tube Test. *Staphylococcus aureus* Oxford was used as the penicillin-sensitive test organism for the determination of penicillinase activity. All penicillin-resistant coagulase-positive *Staph. aureus* strains showed considerable penicillin activity after production of the corresponding prepa-

TABLE I
Comparison of Penicillinase Inhibition and Surface Activity

Substance	Penicillinase activity, $\mu\text{g.}$	Surface tension, dynes/cm., (0.1% aqueous solution)
Penicillinase alone (control)	2.63	—
Penicillinase plus sodium tridecyl-2-sulfate	>30.00	34.9
Penicillinase plus sodium-dodecylbenzenesulfonate	30.00	30.5
Penicillinase plus sodium-laurylsulfate	13.34	27.8

ration. The medium used was nutrient broth. The penicillin used was sodium penicillin G. The horse serum was sterile, without any additive.

The intracellular staphylococci penicillinase was obtained from penicillin-resistant coagulase-positive strains by the method developed by Harper² as modified by Ortel.³ This preparation is a powdery residue obtained after pretreatment with ether and acetone, and is easily suspended in isotonic saline solution.

A total of 0.3 ml. of the corresponding test solution (suspended in distilled water and/or serum) and/or the pure solvent is added to 0.3 ml. of penicillinase suspension (300 $\mu\text{g./ml.}$) and stirred well. After four hours at 37 C. a dilution series (2/3) is made from this mixture by means of a 0.9 per cent saline solution. To every tube containing 0.2 ml. of this dilution series, 0.8 ml. broth, containing both penicillin and the test germ *Staph. aureus* Oxford (bred for 20 hours), is added. The penicillin quantity in each tube amounts to 0.15 $\mu\text{g.}$ and causes a complete inhibition of the growth of the test germ.

After an incubation of 20 hours at 37 C. the findings are read off: The last overgrown tube in the test as well as in the control series indicates the penicillinase concentration that still inactivates 0.15 $\mu\text{g.}$ penicillin. The lower this concentration, the more effective will be the corresponding penicillinase preparation.

TABLE II
Penicillin Sensitivity, in Units/ml., of Resistant Staphylococcus Strains with and Without the Addition of Test Substances (20 $\mu\text{g./Tube}$)

Substance	Serial number of test strains			Surface tension, dynes/cm. (0.1% aqueous solution)
	1	2	3	
Penicillin G sodium (control)	250.0	250.0	250.0	—
Penicillin G sodium				
Plus sodium tridecyl-2-sulfate	0.07	0.15	0.15	34.9
Plus sodium laurylsulfate	2.5	0.62	1.2	27.8
Plus Mersolat H	0.07	0.62	0.31	32.0
Plus Igepon AP	—	0.03	0.03	28.6
Plus Rapidnetzer BASF	0.31	0.31	0.07	26.1
Plus sodium-dodecylbenzenesulfonate	0.15	—	0.31	30.5
Plus Leonil DB	0.62	2.5	—	38.2
Plus Ultravon K	10.0	2.5	—	43.1
Plus Rapidnetzer RBD	0.03	0.07	—	25.8
Plus Lissapol LS	0.62	0.62	—	28.8

TABLE III

Relationship of Surface Activity and Degree of Reduction of Resistance

	Surface tension, dynes/cm. (0.1% aqueous solution)
Effective Compounds	
Sodium tridecyl-2-sulfate	34.9
Sodium laurylsulfate	27.8
Mersolat H	32.0
Igepon AP	28.6
Rapidnetzer BASF	26.1
Sodium-dodecylbenzenesulfonate	30.5
Leonil DB	38.2
Ultravon K	43.1
Rapidnetzer RBD	25.8
Lissapol LS	28.8
Ineffective or Weakly Effective Compounds	
Cocinic acid-ethanolamidesulfate	27.5
Igepon T	28.4
Nekal BX	35.5
Sodium apocholate	41.5
Sodium dehydrocholate	50.1

Sensitivity Test. To falling penicillin concentrations (halving series) a constant quantity of the test germ (penicillin resistant) as well as of the test solution and of the solvent is added in each case, and after an incubation of 20 hours at 37 C., the penicillin concentration of the last tube not overgrown is determined.

RESULTS

A comparison of penicillinase inhibition and surface activity is shown in table I. The minimum penicillinase concentration of a penicillin-resistant strain that is necessary to render 0.15 μ g. of penicillin ineffective is given in μ g. Whereas without addition of inhibitory substances a penicillin activation is attained with 2.63 μ g. of penicillinase, considerably higher dosages are required when such substances are added. A parallel between the degree of penicillinase inhibition and the surface activity of the inhibitory substances is, however, not recognizable in the case of the three substances listed in table I. Thus, sodium laurylsulfate, with the greatest surface activity, had the least inhibitory effect on penicillinase.

Table II shows the reduction of the resistance of penicillin-resistant strains of staphylococci by various test substances in comparison with surface tension. In general, no parallel is seen here between the degree of reduction of resistance and surface activity. Thus sodium tridecyl-2-sulfate, although its surface activity is lower than that of sodium laurylsulfate, reduces resistance more than the latter. However, an analogous behavior to that mentioned in connection with the experiments to table I was to be expected, because an inhibition of penicillinase¹ may be assumed to be the cause of the production of resistance. On the other hand, several other substances, such as the very surface-active Rapidnetzer RBD, show a certain parallelism between both properties: Rapidnetzer renders the resistant *Staphylococcus* especially sensitive.

Table III, which lists resistance-reducing substances and those that produce no or only a minor (not less than 10 μ g.) effect, shows very clearly that there needs

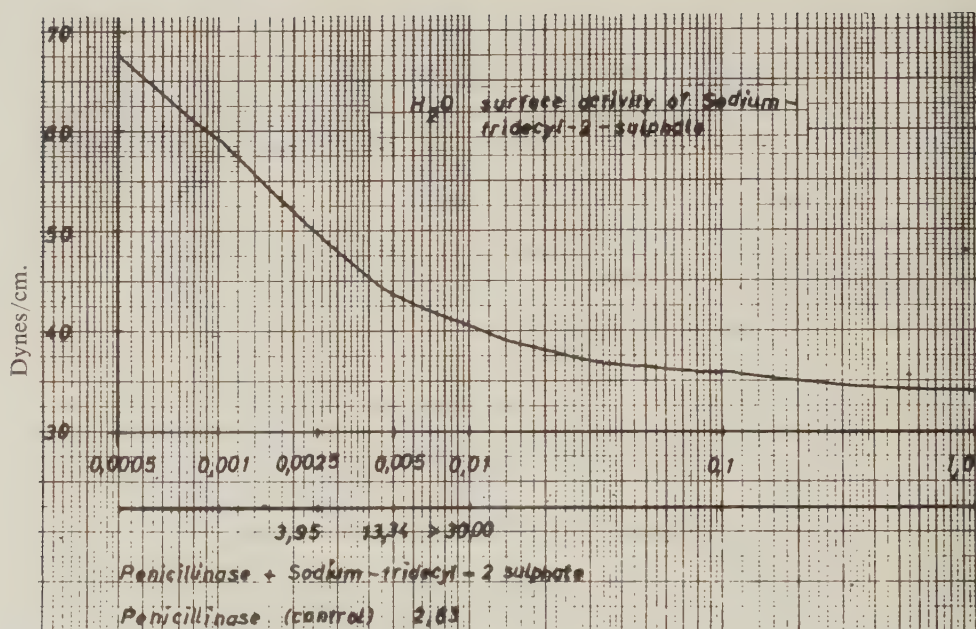


FIG. 1. Dependence upon concentration of the surface activity and the antipenicillinase effect.

to be no parallel between surface activity and the degree to which resistance is reduced. The highly surface-active cocinic acid ethanolamidesulfate was thus found to have little effect on reduction of resistance.

Using as an example the especially effective sodium tridecyl-2-sulfate, we show in figure 1 the dependence on concentration of both the antipenicillinase effect and surface activity. The surface tension of various concentrations of sodium tridecyl-2-sulfate, in dynes/cm., is shown. The illustration shows the quantities of penicillinase, in $\mu\text{g.}$, that are necessary for penicillin inactivation in the corresponding concentrations in the action of sodium tridecyl-2-sulfate.

In this experiment the water value is 73.4 dynes/cm. The addition of 0.0005 per cent of sodium tridecyl-2-sulfate reduces this value to 67.5 dynes/cm. The increase in concentration causes a further decrease in the surface tension of the water, and at 1 per cent it is about 34.2 dynes/cm.

The inhibition of *Staphylococcus* penicillinase was carried out in each case by means of a 0.01, 0.005, and 0.0025 per cent (final concentration) aqueous solution. At 0.0025 per cent the degree of inhibition is very low; compared to the control test, the difference amounts to only one tube (3.95 $\mu\text{g.}$, 2.63 $\mu\text{g.}$). With a 0.01 per cent solution of sodium tridecyl-2-sulfate, more than 30 $\mu\text{g.}$ is required for the inactivation of penicillin (in one tube the penicillin quantity is 30 $\mu\text{g.}$).

Figure 2 shows the surface activity of sodium tridecyl-2-sulfate in distilled water and horse serum of various concentrations. The degree of penicillinase inhibition is given for each individual case.

The aqueous solutions of sodium tridecyl-2-sulfate show a much more intense surface activity than the corresponding serum solutions. It is of interest, from a qualitative point of view, that there is a parallel development between surface activity and effectiveness in reduction of resistance, especially since the sodium

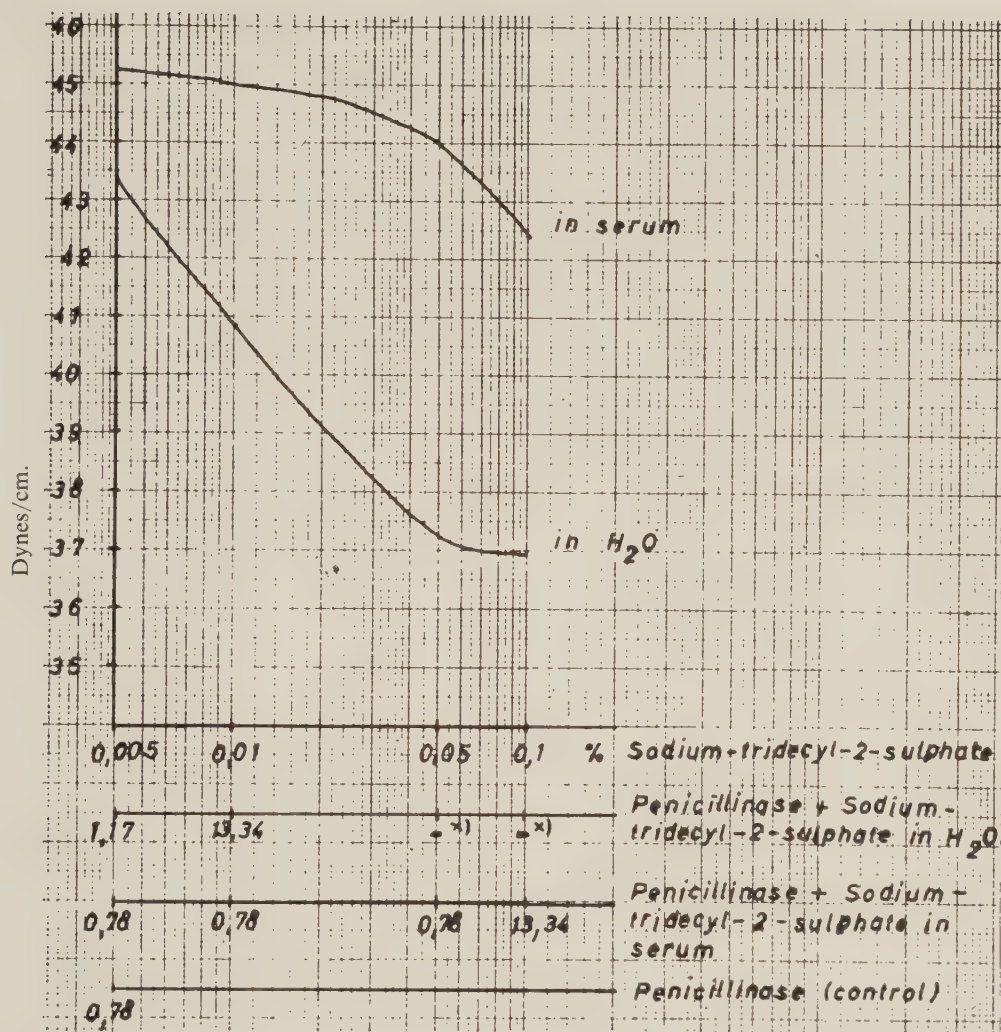


FIG. 2. Surface activity and penicillinase inhibition of sodium tridecyl-2-sulfate, dissolved in distilled water and serum. Such penicillinase quantities are given in $\mu\text{g.}$ as are required in the case of the action of sodium tridecyl-2-sulfate in the corresponding concentrations for the inactivation of penicillin.

tridecyl-2-sulfate dissolved in water is much more effective than that dissolved in serum. In this connection, a 0.01 per cent aqueous solution produced the same inhibitory effect as a 0.1 per cent serum solution. In regard to concentration, we thus find an efficacy that differs by one tenth power, whereas the difference in the surface tension of the two solutions is not very great. (The penicillinase preparation of this test is not identical with that given in tables I and III and its effect is greater than that of the previous preparation. The minimum penicillin inactivation dose is 0.78 $\mu\text{g.}$)

The corresponding control tests, without penicillin and penicillinase and sodium tridecyl-2-sulfate in aqueous solution versus sodium tridecyl-2-sulfate dissolved in serum, show in these concentrations a self-inhibition of the tubes in the test series. We are therefore not able to give the degree of penicillinase inhibition.

DISCUSSION

The results obtained by previous investigations carried out by the authors show it to be possible, by the addition of surface-active substances, to return highly resistant strains of staphylococci to the activation range of penicillin, i.e., to make them sensitive. After penicillinase is inhibited in a similar manner by these substances, there appears to be a certain connection between penicillinase inhibition on the one hand and the reduction of resistance on the other. It appears certain, however, that there is no parallel development between the surface activity of the tested substances and the reduction of resistance as a rule, especially since highly surface-active substances have proved to be ineffective in reduction of resistance. There is, however, a certain parallelism in the case of some substances.

However, these tests, which were carried out *in vitro*, allow no definite conclusion to be drawn as to a possible *in vivo* application, particularly as these substances cannot be administered parenterally because of their surface activity. Besides, it was found that serum cancels the inhibition of penicillinase for the most part. A possibility of success would exist only if it were possible to find substances that can be administered parenterally and are not inhibited in the serum, although other factors are certain to play an important part.

SUMMARY

We were able to show that highly resistant strains of staphylococci behave *in vitro* like normally nonresistant strains, if the inhibition of the penicillinase is correspondingly high. As these penicillinase-inhibiting substances, which themselves produce no disturbing inhibitory effect under suitable experimental conditions, are anion surface-active substances such as sodium tridecyl-2-sulfate and sodium-dodecylbenzenesulfonate, the question of a relationship between the inhibition of penicillinase and the surface activity of these substances has arisen and has been investigated. All of the effective substances that we tested have a surface activity of between 25.8 and 50.2 dynes/cm. There does not appear to be a direct connection between the degree of penicillinase inhibition, as well as neutralization of associated resistance, and the intensity of surface activity. Numerous surface-active substances of the anion type could be found showing almost no inhibition of penicillinase.

With effective substances, the inhibition of penicillinase, as well as surface activity, depend on concentration. It was also found that addition of serum leads to a reduction of surface activity and, at the same time, to a reduction of the antipenicillinase action.

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A Controlled Blind Study of Pneumococcal Pneumonia Treated with Tetracycline and Tetracycline Plus 6-Methyl Prednisolone

J. KIRBY, G. POLIS, AND M. J. ROMANSKY

*Department of Medicine, George Washington University School of Medicine, and
George Washington University Medical Division, District of Columbia General
Hospital, Washington, D. C.*

The value of adrenal steroids as adjuvants to the chemotherapy of bacterial infections is equivocal. The general use of these agents in uncomplicated bacterial diseases is not advocated. Certain infectious diseases, such as typhoid fever, brucellosis, severe tetanus, and Rocky Mountain spotted fever, may be associated with a highly febrile toxic course. There is evidence that patients with these diseases may benefit from the administration of adrenal steroids along with appropriate and adequate antibiotic coverage.¹⁻⁵ Experimental studies and clinical data have shown that the adrenal steroids, used alone or with inadequate antibiotic coverage in infections, may result in dissemination of the infectious process, occasionally with fatal results.^{6,7} Surprisingly few controlled studies have been reported in infectious diseases on the use of adrenal steroids with adequate antibiotic coverage. One controlled study on an entity with a relatively predictable outcome, pneumococcal pneumonia, has been described.⁸ It is not the purpose of this paper to review the relationship of adrenal steroids to systemic infections, which has been done by one of us and others,^{6,7} but to present the data on a controlled blind study of pneumococcal pneumonia treated with tetracycline alone and tetracycline plus 6-methyl prednisolone.*

METHODS

The diagnosis of pneumonia in the 42 patients in the study was confirmed by clinical, roentgenographic, and laboratory evidence. Before starting therapy, direct smears of the sputum were stained by the Gram method. Sputum cultures, as well as aerobic and anaerobic blood cultures, were obtained before therapy was instituted. The study was composed of two groups of patients, 21 in each group. The control group received tetracycline alone and the other received tetracycline plus 6-methyl prednisolone. The characteristics of these patients are summarized in table I. As noted in the table, the sputum smears of all 42 patients had large numbers of gram-positive diplococci morphologically resembling the pneumococcus. On discharge, patients were requested to return to the clinic in two weeks for further clinical and roentgenographic evaluation.

The tetracycline and tetracycline-steroid capsules were identical in appearance and were given under a code number. Each capsule contained 250 mg. of tetracycline or 250 mg. of tetracycline plus 4 mg. of 6-methyl prednisolone. The identity of the capsules was unknown until the end of the study. Patients in both groups

* The trade name of The Upjohn Co. for tetracycline is Panmycin; for tetracycline plus 6-methyl prednisolone, Panmycin plus Medrol. These drugs were supplied through the courtesy of this firm.

received 2 Gm. of tetracycline per day, 500 mg. every six hours per os for a minimum of five days or until they were afebrile for a period of 48 hours. Those patients receiving steroids were given a total of 32 mg. of 6-methyl prednisolone on the first day, 16 mg. on the second and third days, and 8 mg. on the fourth day.

RESULTS

The most striking result of the study was the more rapid defervescence in the tetracycline plus 6-methyl prednisolone-treated patients. This defervescence is illustrated in figure 1 and summarized in table II. In this group, 3 patients had temperatures of 99 F. on admission and did not become febrile after the institution of therapy. Of the remaining 18 patients, 13 or 72.2 per cent were

TABLE I
*A Controlled Blind Study of Pneumococcal Pneumonia:
Characteristics of the 42 Patients in the Study*

	Number of patients on tetracycline (21)	Number of patients on tetracycline plus 6-methyl prednisolone (21)
Type of pneumonia		
Lobar pneumonia	13	21
Bronchopneumonia	6	—
Negative chest roentgenogram	2	—
Age, years		
15-30	7	4
31-50	6	10
51-77	8	7
Women	5	7
Men	16	14
Coexisting conditions		
Arteriosclerotic heart disease	1	1
Chronic alcoholism	1	3
Asthma	—	3
Hypertensive cardiovascular disease	3	3
Bronchiectasis	2	1
Duration of symptoms before admission, days		
1-2	11	11
3-4	7	5
5-7	3	5
Temperature on admission		
103-105 F.	12	7
101-102 F.	9	11
99-100 F.	—	3
White blood cells/cu. mm. on admission		
10,000 or less	9	4
11,000-20,000	9	13
21,000-30,000	3	4
Gram stains positive for pneumococci	21	21
Sputum cultures		
Pneumococci	6	3
Nonspecific	15	18
Positive blood cultures for pneumococci	None	2

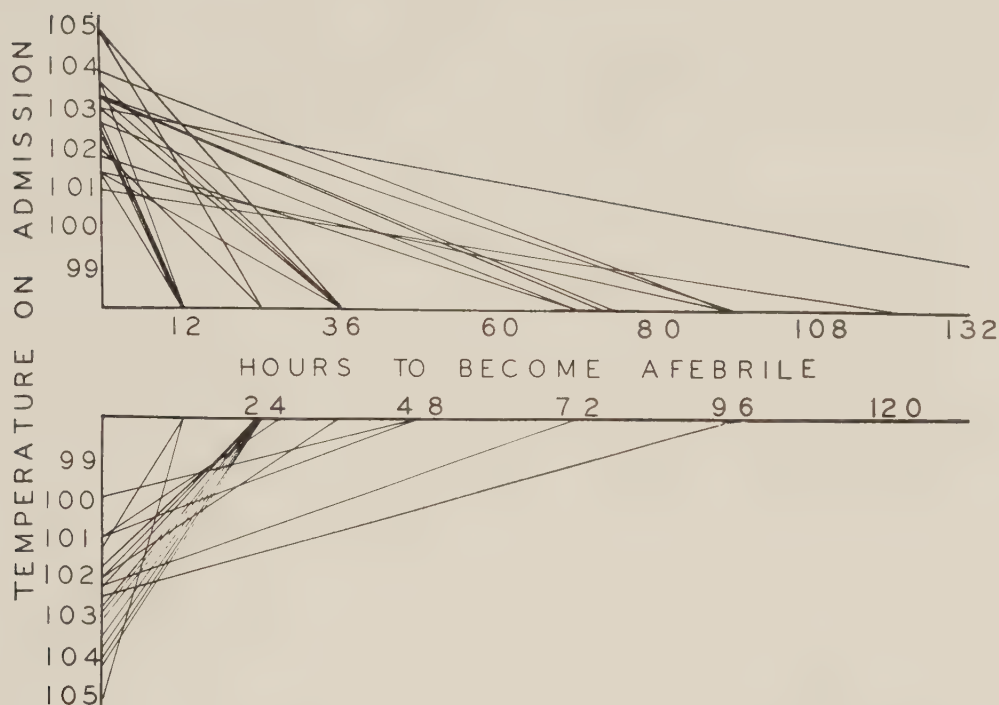


FIG. 1. Defervescence of fever in a controlled blind study of pneumococcal pneumonia. Top: 21 patients treated with tetracycline. Bottom: 21 patients treated with tetracycline plus a steroid, 6 methyl-prednisolone. Three of these patients had a temperature of 99 F. on admission.

afebrile by 24 hours after initiation of therapy and 3 additional patients became afebrile within 48 hours. Hence, 16 patients or 88.8 per cent were afebrile in 48 hours after the institution of antibiotic-steroid therapy. The 2 remaining patients became afebrile in 72 and 96 hours.

In the control group of 21 patients, only 7 or 33.3 per cent were afebrile 24 hours after therapy was begun. Five more were afebrile in 36 hours and no additional patients were afebrile at the end of 48 hours. Thus, a total of 12 patients or 57.1 per cent were afebrile within 48 hours after the therapy was instituted. Of

TABLE II

A Controlled Blind Study of Pneumococcal Pneumonia: Subsidence of Fever by Hours

Hours	Number of patients on tetracycline (21)	Number of patients on tetracycline plus 6-methyl prednisolone (21)
12	5	2
24	2	11
36	5	1
48	—	2
72	2	1
84	2	—
96	3	1
120	1	—
124	—	—
148	1	—

TABLE III

*A Controlled Blind Study of Pneumococcal Pneumonia:
Degree of Resolution of Pneumonia in the 42 Patients*

	Number of patients on tetracycline (21)	Number of patients on tetracycline plus 6-methyl prednisolone (21)
No change or minimal clearing	4	2
Marked resolution	11	10
Complete resolution	4	9
Negative on admission	2	—

the remaining patients, 2 became afebrile in 72, 2 in 84, and 3 in 96 hours. One patient became afebrile on the fifth day and another on the seventh day.

In the tetracycline-steroid-treated group, temperature elevations occurred in 2 patients after afebrile periods of one and two days respectively. The temperature did not exceed 101 F. and occurred while the patients were receiving the 6-methyl prednisolone. The patient who had a recurrence of fever one day after becoming afebrile subsequently developed a pleural effusion and had an unresolved pneumonia. This patient will be discussed in more detail. The other patient suffered no complications. No recurrence of fever was noted after the 6-methyl prednisolone was discontinued.

In the control group, in 3 patients a single spike in fever occurred after afebrile periods of 11 and 3 days respectively in 2 patients and one day in a third. This single spike in the temperature course of the 3 control patients did not exceed 102 F. and was an isolated finding not associated with a suppurative complication or clinical relapse.

A comparison of the roentgenographic results in the two groups (table III) was not so striking as the response in fever. In the tetracycline plus 6-methyl prednisolone-treated group, 9 patients had complete resolution of the pneumonia as shown by chest roentgenogram. This resolution took place between the third and eleventh days after therapy was instituted. Ten patients showed significant resolution of the pneumonic process during the period of hospitalization. Follow-up study was made in 1 patient in this group and the chest roentgenogram was completely clear 22 days after discharge. In 2 patients no resolution had taken place by the time of discharge. Follow-up was available in 1 patient who was readmitted eight weeks later and died. This patient is discussed in more detail under "Complications."

In the control group, 4 patients had complete clearing of the pneumonia by the fourth, seventh, ninth, and tenth days respectively after therapy was begun. Significant resolution of the pneumonia as shown by roentgenogram was observed in 11 patients. Follow-up examination was available in 3 patients in this group. Complete resolution had occurred in 1 patient who returned 50 days after discharge, nearly complete resolution in another in 26 days, and no significant change in a third who returned 11 days after discharge. Of the 4 patients classified as having no change in the chest roentgenogram during their period of hospitalization, follow-up examination was available in 1. In this patient no significant resolution was noted 22 days later.

No significant difference in laboratory findings was observed in either group.

COMPLICATIONS

Suppurative complications such as empyema, meningitis, and pericarditis did not occur in either group. One patient in the tetracycline control group had persistent pleuritic pain for three days after therapy was instituted. No further complications occurred in this patient.

A minimal pleural effusion not requiring thoracentesis with subsequent spontaneous reabsorption occurred in 1 patient in the control group and in 1 patient in the tetracycline-steroid group.

No deaths occurred in either group during the course of the study.

One patient in the tetracycline-steroid-treated group died eight weeks after discharge. This patient was a 31 year old Negro man admitted with a right lower lobe pneumonia and blood cultures positive for a pneumococcus. He was a known chronic alcoholic with marked hepatomegaly and also had epilepsy as a sequel to the evacuation of a subdural hematoma five years prior to this admission. The patient became afebrile three days after tetracycline plus 6-methyl prednisolone therapy was instituted. However, a single spike in temperature to 101 F. occurred on the fifth day. The 6-methyl prednisolone-tetracycline combination was discontinued on the sixth hospital day. He received an additional four days of tetracycline, 2 Gm. per day, and remained afebrile. Despite his asymptomatic and afebrile course, physical examination and chest roentgenogram revealed fluid in the right pleural space on the fifteenth hospital day. Thoracentesis at this time yielded 300 ml. of straw-colored fluid, which was sterile on culture. No reaccumulation of fluid occurred roentgenographically. There was no significant change in the right lower lobe infiltrate by the nineteenth hospital day. The patient was afebrile and asymptomatic when discharged to follow-up in the outpatient clinic on the twentieth hospital day with a diagnosis of slowly resolving pneumonia. He failed to return to the clinic and was readmitted eight weeks after discharge with the symptoms, signs, and roentgenographic findings of an active right lower lobe pneumonia and aortic insufficiency. The patient died 24 hours after admission despite adequate penicillin therapy. Permission for post-mortem examination was not obtained.

Complications that could be directly attributed to the steroid, such as gastrointestinal hemorrhage, severe electrolyte imbalance, or disturbances of mood and orientation, were not observed.

DISCUSSION

In this study, the 21 patients treated with 6-methyl prednisolone in decreasing amounts over a five day period with 2 Gm. tetracycline per day showed a more rapid defervescence than a similar group treated with the same amounts of tetracycline alone. No "recurrence" or "rebound" of fever was noted on the discontinuance of the 6-methyl prednisolone, nor was there any instance of hypothermia with the initial dosage of steroid therapy, as was observed by Wagner et al⁸ in a similar study. The roentgenographic response in the tetracycline-6-methyl prednisolone group, as compared to the control group, was not so conclusive as the defervescence of fever. Despite the larger number of patients in the tetracycline-6-methyl prednisolone-treated group showing complete resolution of the pneumonia,

the actual number of days to complete clearing had essentially the same distribution as the control group. No significant conclusions can be drawn from a comparison of those patients classified as having significant clearing of the chest roentgenogram due to the approximately equal numbers in each group and the lack of follow-up. During the period of hospital observation, 4 patients in the control group and 2 patients in the tetracycline-6-methyl prednisolone group failed to show improvement roentgenographically. These results are considered inconclusive due to the small numbers in this category.

Complications in both groups were minimal except for the incident in the tetracycline-steroid group of the patient who developed a 300 ml. pleural effusion on the fifteenth day and whose right lower lobe pneumonia failed to respond despite clinical remission. This patient's death eight weeks later from a recurrent severe right lower lobe pneumonia and aortic insufficiency is somewhat difficult to attribute directly to the short-term course of 6-methyl prednisolone some two months before, but it does introduce a precautionary note.

Lepper and Spies,⁹ in a controlled study using appropriate antibiotic therapy with hydrocortisone in the treatment of meningococcal and *Hemophilus influenzae* meningitis, concluded that no benefit could be expected from the routine employment of steroids in these diseases.

Wagner et al⁸ in a controlled study of 113 patients with pneumococcal pneumonia, 52 of whom received penicillin and hydrocortisone and 61 of whom received penicillin alone, felt that "the symptomatic benefit of hydrocortisone and the absence of any evidence to indicate the aggravation of the infectious process in pneumococcal pneumonia justified further cautious exploration of adrenal steroids as adjuvants to specific antibiotic therapy."

It would appear from this study that there is no indication for steroids in conjunction with antibiotics in the treatment of uncomplicated pneumococcal pneumonia but additional controlled studies are needed on the effect of adrenal steroids and antibiotics in patients who are highly febrile and "toxic."

SUMMARY AND CONCLUSIONS

A controlled blind study was carried out on 42 patients with pneumococcal pneumonia; 21 patients received tetracycline plus 6-methyl prednisolone and a similar group of 21 patients received tetracycline alone. As might be expected, the tetracycline plus 6-methyl prednisolone-treated group showed a more rapid defervescence than the control group. Comparison of the roentgenographic findings in both groups was inconclusive. Complications were minimal in both groups with the exception of 1 patient in the tetracycline plus 6-methyl prednisolone-treated group who died eight weeks after his initial hospitalization with a right lower lobe pneumonia and aortic insufficiency. It is difficult to attribute his death to a short course of 6-methyl prednisolone some two months before, but it does introduce a precautionary note.

Complications directly attributable to the 6-methyl prednisolone did not occur.

Despite the more rapid defervescence in the 6-methyl prednisolone-treated group, there is no indication for adjunctive steroid therapy in uncomplicated pneumococcal pneumonia.

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The Effect of Long-Term Tetracycline Therapy on Chronic Bronchitis. A Preliminary Report

PHILIP S. NORMAN, EDWARD W. HOOK, ROBERT G. PETERSDORF,
LEIGHTON E. CLUFF, MALCOLM C. GODFREY, JOHN C. RIBBLE, AND
ALLAN H. LEVY

Department of Medicine, Johns Hopkins University and Hospital, Baltimore, Md.

Patients with chronic bronchitis are highly susceptible to intercurrent infections of the respiratory tract, and these exacerbations culminate in progressive ventilatory insufficiency. Recently Brumfitt et al¹ demonstrated bronchoscopically that while the lower respiratory passages are normally sterile, the bronchi of patients with chronic bronchitis are consistently colonized by microorganisms. *Hemophilus influenzae* has usually been the offending pathogen cultured in the sputum of these patients,²⁻⁴ and Hers and Mulder⁵ demonstrated these microbes deep within the mucosa of the bronchi at autopsy. This organism, as well as *Diplococcus pneumoniae*, has also been frequently implicated in acute exacerbations.⁶ In order to prevent both acute flare-ups and to diminish colonization of bronchi by bacteria, suppressive antibacterial therapy has been given to these patients, with *Hemophilus* the primary target. Because *H. influenzae* is a normal inhabitant of the mouth and pharynx, it seems unlikely that it can be eradicated completely; however, a significant reduction of numbers might be clinically useful. Antibiotic sensitivity studies indicated that various strains of this organism are regularly susceptible to chloramphenicol and tetracycline,⁶ and the effect of several dosage schedules has been the subject of controlled studies.

McVay and Sprunt⁷ prevented an appreciable number of exacerbations in 21 patients with chronic bronchitis by the administration of 500 mg. chlortetracycline per day. Moyes and Kershaw,⁸ in a double-blind study, demonstrated that daily administration of 750 mg. tetracycline during the winter months produced general improvement in the status of chronic bronchitis and reduced the number and severity of exacerbations.

Elmes et al⁹ found that 1 Gm. oxytetracycline per day for five days at the first sign of a cold reduced the severity of exacerbations of chronic bronchitis. Franklin and Garrod¹⁰ administered chloramphenicol to 36 patients with bronchiectasis in doses of 20 mg./Kg. three times a day. This dose consistently diminished the volume of sputum and eliminated pathogenic bacteria. The investigation, however, was discontinued when one patient died of aplastic anemia. Cherniak et al¹¹ gave 17 patients with chronic bronchitis and bronchiectasis 2 Gm. of tetracycline per day and found the incidence of both mild and severe exacerbations to be less than in a control group. The frequency of isolation of pneumococci and coagulase-positive staphylococci was significantly reduced, but isolations of *H. influenzae* were only

This study was performed under a contract with the U.S. Army Chemical Corps, Fort Detrick, Frederick, Md.; it was aided by a grant from Chas. Pfizer & Co., who also furnished the tetracycline; and supported in part by training grant 2E-9, National Institute of Allergy and Infectious Disease.

slightly less frequent. Dosage had to be reduced in 4 patients because of adverse reactions to the drug.

In general, these controlled studies of the use of antibiotics over prolonged periods have indicated a beneficial effect on the course of chronic bronchial infection. However, the series was small, and the difference between control and treated groups was not great. Furthermore, the conclusion that the patients given antimicrobials were better depends upon the assumption that, if untreated, they would have had as much illness as the control group. The variability in the course and severity of this chronic disease makes the collection of well-matched groups of patients difficult. Consequently, in the present study patients served as their own controls, and their clinical and laboratory status was compared in successive three month periods during which they received either tetracycline, 1 Gm./day, or placebo. Although the study is still in progress, a preliminary evaluation of the results was made after 12 months and tends to confirm the beneficial effect of tetracycline on the course of chronic bronchitis. The number of acute exacerbations was somewhat reduced, cough became less severe, and the quantity of sputum diminished. As in other studies, there was some reduction in the frequency of isolation of *Hemophilus* and pneumococci from the sputum.

MATERIALS AND METHODS

Patients. Chronic bronchitis was considered to be present if there was a history of chronic cough of at least one year's duration, associated with expectoration of mucoid sputum for at least three months during the year and with periodic exacerbations during which sputum became purulent. Patients selected for the study ranged from those with relatively little disability to severe pulmonary cripples with continuous cough and copious sputum, in whom an exacerbation was a life-threatening illness. Tuberculosis was ruled out by means of several cultures for acid-fast organisms. No attempt was made to exclude those, who, in addition to chronic bronchitis, also had emphysema, bronchiectasis, or asthma.

Patients were divided into mild and severe groups. The disease was considered to be mild if the patient was able to lead an almost normal life apart from an occasional exacerbation. On the other hand, if his existence was sufficiently affected by the illness as to cause him to lose time from work or if total disability was present, the disease was termed severe.

Treatment Plan. Patients were required to have had at least one month without antibiotic therapy before being placed on the study. Treatment consisted of either tetracycline,* 1 Gm./day in divided dosage, or indistinguishable placebos. Patients were assigned to the drug or placebo group at random, and after three months the schedule was reversed for a second three months. Tetracycline or placebo was again selected at random for the third three month period, and the alternate drug was given during the fourth period. Symptomatic therapy with bronchodilators, expectorants, or adrenal cortical hormones was given as indicated.

Evaluation. Each patient had a medical history and complete physical examination at the beginning of the study. Other studies included chest and sinus roentgeno-

* The trade name of Chas. Pfizer & Co. for tetracycline is Tetracyn.

TABLE I

Clinical Status During Each 3 Month Period

Status	Placebo	Tetracycline
Better	11	21
No change	15	11
Worse	13	7

grams, complete blood counts, and tuberculin skin tests. On the morning of each clinic visit, patients were instructed to collect in a sterile container all sputum expectorated between the time of arising until arrival at the clinic. The volume of sputum was measured; the entire sample was homogenized for one hour at 37 C. with an equal volume of 1 per cent pancreatin buffered at pH 7.6, and quantitative plate counts were performed for each organism recognized in the sample. Pulmonary function was tested on each visit by measurement of total vital capacity and 1 second vital capacity.

After the start of therapy patients were seen at monthly intervals. In addition to measurement and culture of sputum and the pulmonary function studies described, a history was obtained with particular emphasis on the severity of cough, the quantity and appearance of sputum produced, exercise tolerance, acute respiratory symptoms, and toxic reactions to the drug. The patient was weighed and the chest examined. An acute exacerbation was defined by the presence of increased cough and purulent sputum for three consecutive days coupled with sufficient disability to prevent or impair work.

RESULTS

Clinical Observations. At the time of the preliminary evaluation 30 patients had completed at least one three month period of treatment, and all of these are included in this report. Nine patients have been studied for four periods, 7 patients for three periods, 7 patients for two periods, and 7 patients for only one period. Thus, a total of 78 treatment periods are available for analysis, 39 periods with tetracycline and 39 periods with placebo.

The over-all clinical status of the patients is shown in table I. These results represent the evaluation of the attending physician on clinical grounds, without refer-

TABLE II

Number of 3 Month Periods with Acute Exacerbations of Chronic Bronchitis

	Placebo	Tetracycline
No exacerbation	17	23
One exacerbation	19*	14†
Two exacerbations	3	2

* Three with pneumonia; 1 with sinusitis.

† Two with pneumonia; 1 fatal.

TABLE III

Quantity of Sputum During Each 3 Month Period

	Placebo	Tetracycline
Decrease	14	19
No change	16	12
Increase	9	8

ence to the bacteriological or pulmonary function data. Patients were considered "improved" during twice as many tetracycline periods as placebo periods; conversely, deterioration in clinical status occurred twice as often on placebo as on the antimicrobial agent. There was a considerable group in the middle that showed no change. With one exception, there was no dramatic improvement during tetracycline therapy. This patient was a 55 year old man with severe emphysema and respiratory insufficiency, long-standing cough, and profuse sputum. He had suffered two severe exacerbations requiring hospitalization during the previous winter. After three months of therapy with tetracycline, sputum production became almost nil, and the cough disappeared. The patient has been followed for a year and has had no exacerbations, even of mild degree; emphysema and exercise intolerance remain.

In contrast, failure of chemoprophylaxis is illustrated by a 60 year old woman with chronic bronchial asthma, which had progressed to a stage of severe bronchitis characterized by disabling cough and purulent sputum. During the second period of therapy with tetracycline, she began to harbor antibiotic-resistant staphylococci in the sputum. Subsequently, she developed an acute bronchial infection with severe asthma, which required hospitalization. After several weeks of intensive therapy with multiple antibiotics, steroids, and bronchodilators, she died in status asthmaticus.

Table II shows the number of treatment periods during which acute exacerbations occurred. Patients receiving tetracycline had a few more periods free of exacerbations than controls. Three severe flare-ups requiring hospitalization began during administration of placebo, and two exacerbations, one of which was eventually fatal, started during tetracycline therapy.

Tables III and IV show the effect of antimicrobial prophylaxis on the severity of cough and quantity of sputum. These results, based on the subjective impressions of each patient, tended to demonstrate some improvement on tetracycline and slight deterioration on placebo, but this trend was not marked.

No significant toxic reactions to this dose of tetracycline were reported. Thirty of the 39 periods on tetracycline were free of adverse side effects, while 33 of 39

TABLE IV

Severity of Cough During Each 3 Month Period

	Placebo	Tetracycline
Better	11	16
No change	18	17
Worse	10	6

placebo periods were so described. Symptoms that could have been the result of treatment with tetracycline, such as gastrointestinal distress, diarrhea, or itching, were reported as often in association with placebo as with tetracycline. One patient developed recurrent urticaria and angioneurotic edema while taking the antibiotic. Not only tetracycline but also other symptomatic medications were discontinued and treatment with prednisone instituted. The hives subsided completely during the ensuing two months after which the patient took full doses of tetracycline without ill effects.

Our study confirms the findings of others that *H. influenzae* is an important pathogen in chronic bronchitis. Administration of tetracycline reduced but did not eliminate the number of cultures positive for *Hemophilus*. Furthermore, quantitative examination of each positive culture revealed at least as many organisms during

FIG. 1. Quantitative sputum bacteriology in patients with chronic bronchitis. Each symbol indicates isolation on a single culture. Nonhemolytic staphylococci, *Neisseria* species, diptheroids, and streptococci were considered normal flora.

TABLE V

*Incidence of Significant Respiratory Pathogens
in Cultures of Sputum*

	Pretreatment	Tetracycline	Placebo
No. specimens	38	100	97
Per cent positive			
<i>H. influenzae</i>	55	29	39
<i>D. pneumoniae</i>	13	2	16
<i>Staph. aureus</i>	16	11	11
Enterobacteriaceae	34	31	28
Fungi	5	8	4

tetracycline therapy as prior to treatment. In patients receiving placebo, the incidence of cultures positive for *H. influenzae* increased but not to the pretreatment level. This may be due in some patients to suppression of the growth of the organisms by tetracycline given during the antecedent treatment period.

Pneumococci were effectively eradicated from the sputum by prophylaxis with tetracycline. Whether this affected the patients' clinical course is a moot point.

Hemolytic staphylococci were cultured infrequently from specimens of sputum and, when present, were relatively few in number. Treatment with tetracycline did not appreciably reduce the rate of colonization with staphylococci. More important, however, the small doses of the antibiotic administered did not increase the frequency of isolations of staphylococci from sputum.

Although enterobacteriaceae, encompassing *Escherichia coli*, *Klebsiella* species, *Proteus* species, and *Pseudomonas aeruginosa* were found in approximately one third of the samples, these organisms were not influenced by tetracycline. Finally, colonization by fungi did not present a problem.

Pulmonary Function. The one minute and total vital capacity was not altered during the period of treatment, and, for the sake of brevity, the detailed data are not presented in this preliminary report.

COMMENTS

At this early stage of the study, it is not possible to evaluate the data employing each patient as his own control, since a number of patients have completed only one or two study periods. For the present analysis therefore all control and treatment periods have been combined into two large groups. Eventually, it should be feasible to compare a sufficient number of control and treatment periods in an individual patient to assess the effect of chemoprophylaxis more definitively.

On the basis of the available data, it appears that prophylactic therapy with tetracycline was of some benefit in patients with chronic bronchitis, regardless of the subjective index used to measure improvement—general well-being, severity of cough, quantity of sputum, and occurrence of acute exacerbations. This effect was noted in patients with both mild and severe disease. On the other hand, this mode of therapy was not a panacea, and the disease progressed in a number of patients despite

chemotherapy. In general, however, symptoms were somewhat more severe in untreated patients.

One factor which may obfuscate the value of tetracycline is the tendency for patients with chronic bronchitis to improve spontaneously during the summer. Our patients were no exception and, in general, improved during the summer whether receiving tetracycline or placebo. By continuing the study for several more years, enough data should be accumulated to compare the effects of therapy during the summer and winter months. It is conceivable that limitation of chemotherapy to periods of intemperate weather might be as beneficial as continuing treatment throughout the year. Modification of the program toward "seasonal treatment" would also considerably decrease its expense.

A second possible cause of erroneous interpretation of these data is failure to consider the order in which therapy was administered. For example, it seems conceivable that a patient could receive sufficient benefit from a three month course of tetracycline to carry over into the subsequent period of treatment with placebo. The results would be different, of course, if the order of therapy were reversed. At present, not enough patients have been treated continuously to evaluate this possible source of error.

Whereas there was little change in the bacterial flora of the sputum in response to tetracycline, the reduction in the number of cultures positive for *H. influenzae* and *D. pneumoniae* may be significant. It would be worthwhile to confirm the results of these sputum cultures with bronchoscopic aspirations, since Brumfitt et al¹ demonstrated that the flora of the lower bronchial tree, free from oral contamination, may differ appreciably from that in expectorated sputum. It is conceivable that organisms colonizing the peripheral bronchi were more effectively suppressed than was reflected in cultures of sputum.

It should be emphasized that this is a preliminary report and that the results described should be interpreted in this light. On the basis of the data obtained thus far, the long-term therapy of chronic bronchitis with broad-spectrum antibiotics shows sufficient promise to deserve further trial.

SUMMARY

Thirty patients with chronic bronchitis were treated with 1 Gm. of tetracycline per day for three month periods alternating with similar periods during which placebo was given.

The patients' clinical status was improved to some degree while they were receiving tetracycline. There were fewer acute exacerbations, and cough and sputum were diminished.

The frequency of isolation of *Hemophilus influenzae* and *Diplococcus pneumoniae* from sputum cultures was reduced by the antibiotic, but the flora was otherwise unchanged. The results indicate that long-term therapy of chronic bronchitis with broad-spectrum antibiotics deserves further trial.

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The Long-Term Management of Chronic Bronchitis with Antibiotics

J. C. GOULD AND J. McC. MURDOCH

*Departments of Bacteriology and Therapeutics,
University of Edinburgh, Scotland*

Chronic bronchitis is a disease with progressive respiratory incapacity characterized by recurrent productive winter cough and increasing dyspnea with effort. The sputum is generally copious, tenacious, and varies from mucoid to mucopurulent or purulent during each winter. Purulence tends to increase as the disease progresses, with destruction of bronchopulmonary tissue leading to gross emphysema.

The precise etiology of this disease is not known. It has been suggested¹ that the primary defect in susceptible persons lies in the abnormal production of excessive mucus by the goblet cells of the bronchial mucosa. This is accentuated by various irritant stimuli, such as industrial, domestic, and tobacco smoke, especially when the winter climate is cold and damp.² The excessive mucus interferes with the ciliary defense mechanism of the bronchi, making them more liable to invasion by micro-organisms. If pathogens are involved infection results causing acute exacerbations of the disease. *Hemophilus influenzae* and the pneumococcus are the organisms most frequently associated with such exacerbations.^{3,4} The prevention of these exacerbations should reduce winter ill-health and prolong life, and in this respect antibiotics active against the pathogens should be of value. For this purpose, the tetracyclines have been successfully used.⁵⁻⁷

This paper reports the results of the use of tetracycline-oleandomycin (Signemycin*) and oxytetracycline (Terramycin†) in the management of patients with chronic bronchitis in Edinburgh during the periods October 1, 1957, to March 31, 1958, and October 1, 1958, to March 31, 1959.

METHODS AND MATERIALS

Twenty-three patients with established chronic bronchitis were studied in the first winter. Clinical and bacteriological observations were made at fortnightly intervals. No treatment was given until January 1, 1958, when 12 patients received, on a chance basis, treatment with tetracycline-oleandomycin, 1 Gm. four times daily, and 11 patients received placebo. Treatment was stopped on March 31, 1958.

Seventy-five similar patients were studied in the second winter, 35 received, by chance, treatment with oxytetracycline, 1 Gm. daily, and 40 received placebo. Treatment was continuous throughout the winter until March 31, 1959. Patients were seen fortnightly either as hospital out-patients or, if necessary, in their homes.

The clinical assessment was based mainly on comparing the amount of time lost from work due to exacerbations of bronchitis by patients receiving no treatment with the amount lost by those taking the antibiotics.

Following each clinical examination, the patients produced an early morning sample of sputum which was bacteriologically examined for its degree of purulence

* The trade name of Chas. Pfizer & Co. for tetracycline-oleandomycin is Signemycin.

† The trade name of Chas. Pfizer & Co. for oxytetracycline is Terramycin.

TABLE I
Bacteriological Finding in 98 Patients before Treatment

Microorganism	Percentage of patients with positive sputum	Number of strains resistant to tetracycline-oleandomycin or oxytetracycline
<i>H. influenzae</i>	78	0
Pneumococcus	72	0
<i>Staph. aureus</i>	24	4

and the presence of *H. influenzae*, pneumococcus, and other pathogenic organisms including *Staphylococcus aureus*.

At no time was either the clinician or the bacteriologist aware of the other's findings unless the clinical condition of the patient made this necessary.

In addition specimens of feces and swabs from the nose of each patient were examined at six week intervals to determine if there had been any change in the nature of the flora or its sensitivity to antibiotics.

RESULTS

Sputum examinations before treatment in both winters showed that *H. influenzae* and pneumococci were present in more than 70 per cent of the 98 patients (table I). *Staph. aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, and coliform strains were isolated much less frequently. The recovery of all of these organisms on culture was not constant in all specimens from individual patients, so that the results given represent the findings in a series of specimens. No strains of *H. influenzae* or pneumococcus isolated before treatment were resistant to tetracycline-oleandomycin or oxytetracycline.

Table II shows that antibiotic treatment is effective in markedly reducing the amount of time lost from work due to exacerbations of bronchitis compared with the time lost by those taking placebo. This applies to both winters but it is noteworthy that in the first winter period October 1, to December 31, 1957, when no treatment was given, the time lost from work by the patients who were later allocated to two treatment groups was the same.

Acute exacerbations of chronic bronchitis were invariably associated with increased purulence of the sputum and the more frequent isolation of *H. influenzae*

TABLE II
Days Lost from Work Due to Bronchitis

Year	Patients receiving placebo				Patients receiving antibiotics			
	Number of patients	Days off work		No. not off at all	Number of patients	Days off work		No. not off at all
		During observation period	During treatment period			During observation period	During treatment period	
1957-1958	11	97	153	2/11	12	90	5	10/12
1958-1959	40	—	358	13/40	35	—	210	22/35

TABLE III

Isolation of Microorganisms from 52 Patients on Inactive and 46 Patients on Active Treatment

Microorganism	Percentage of patients with large numbers in their sputum	
	Antibiotic treatment	Placebo
<i>H. influenzae</i>	9	71
Pneumococcus	13	85

and pneumococci. Antibiotic treatment was effective in reducing the numbers of these pathogens and so preventing acute exacerbations (table III).

No antibiotic-resistant strains of pneumococcus and a small number of tetracycline-resistant strains of *H. influenzae* were isolated from patients during treatment. A limited number of resistant strains of *Staph. aureus* and coliform organisms was recovered but the numbers were not significantly greater from the patients taking antibiotic compared with those who were not. Also there was no evidence of increased nasal or fecal carriage of *Staph. aureus* in the two groups in either winter (table IV).

SIDE EFFECTS

Side effects were relatively few and confined to patients receiving antibiotic treatment (table V). Diarrhea was the most common but never severe enough to justify stopping treatment. Upset of the flora of the upper respiratory and intestinal tracts was minimal.

DISCUSSION

Continuous daily treatment with moderate doses of oleandomycin-tetracycline or oxytetracycline is obviously beneficial for patients liable to acute infective exacerbations of chronic bronchitis. This is most apparent when the amount of winter ill-health in patients taking these drugs is compared with a control group receiving none. Fitness for work is maintained by antibiotic treatment, thus preventing much of the financial loss to the individual which results from this disease. Further, domiciliary or out-patient management is preferable to hospital admission with its potential danger of superinfection with strains of *Staph. aureus* frequently resistant to the tetracyclines.⁸ For this reason, the patients in these trials did not have gross upset of their surface flora and oxytetracycline-resistant strains of *Staph. aureus* were not numerous either in the sputum, anterior nares, or bowel. In none of the patients were these organisms associated with clinical deterioration. Small numbers

TABLE IV

Percentage of Patients with Tetracycline-Resistant Strains During Treatment

Organism	Sputum	Nares	Feces
<i>H. influenzae</i>	8	—	—
Pneumococcus	0	—	—
<i>Staph. aureus</i>	15	8	3

TABLE V
Side Effects (1958-1959)

Side effect	Patients receiving antibiotic	Patients receiving placebo
Nausea	1	2
Diarrhea*		
Persistent		
Loose	1	0
Watery	2	0
Transient		
Loose	5†	0
Watery	0	0
Abdominal colic	1	0
Black tongue	1	0
Pruritus ani	1	0
Rash	1	1
Vomiting	0	1

* Loose stools = frequent ($\times 4$ approx.) partially formed; watery stools = frequent ($\times 6$ approx.) with no formed element; persistent = diarrhea throughout treatment; transient = diarrhea during initial 4 to 8 weeks of treatment.

† Five patients required temporary withdrawal or reduction of treatment for a few days because of diarrhea in patients receiving antibiotics.

of *H. influenzae* became resistant to oxytetracycline but this did not influence the clinical response to treatment.

These trials have also confirmed that both *H. influenzae* and the pneumococcus are the microorganisms most frequently associated with purulence of the sputum and acute exacerbations of the disease. Moreover, the success of antibiotic treatment was demonstrated by the disappearance of pus and these organisms from the patients' sputum. This emphasizes the important role of *H. influenzae* and the pneumococcus in chronic bronchitis.

Side effects amounted to moderate diarrhea in only 8 patients so treated, but this did not necessitate any change in the treatment.

We conclude that the long-term continuous administration of oxytetracycline or tetracycline is effective and is in no way dangerous where chronic bronchitic patients can be treated at home.

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Aspartocin. I. Production, Isolation, and Characteristics

A. J. SHAY, J. ADAM, J. H. MARTIN, W. K. HAUSMANN, P. SHU, AND
N. BOHONOS

*Biochemical Research Section, Lederle Laboratories Division,
American Cyanamid Co., Pearl River, N. Y.*

Aspartocin is an acidic polypeptide antibiotic with a fatty acid moiety. This new antibiotic was given this name because of its high content of aspartic acid.

Two soil isolates identified as *Streptomyces griseus* var. *spiralis** and *Streptomyces violaceus** have been found to produce aspartocin. This antibiotic is primarily active against gram-positive bacteria.¹ In mouse tests, it protects animals infected with gram-positive bacteria² or *Trypanosoma equiperdum*.³ It has also been found to have growth-promoting properties in the chick.⁴ This antibiotic is readily differentiated from amphomycin⁵ by paper chromatography and paper electrophoresis.

It is the purpose of this paper to describe processes for producing aspartocin and some of the chemical features of the molecule.

ASSAY AND FERMENTATION

A strain of *Bacillus subtilis* grown on nutrient agar at pH 7.8 was used for bioassay by the agar plate diffusion method. Samples were diluted in 1 per cent ammonium carbonate solution. Whole broth samples were adjusted to pH 9.0 to 10.0 prior to separation of mycelial growth.

A mutant strain of *S. griseus* var. *spiralis* was grown under aeration and agitation in a medium with the following composition: Proto-peptone (Wilson no. 159), 30 Gm.; refined molasses, 20 Gm.; Edamin protein hydrolysate, 5 Gm.; hydrated magnesium sulfate, 5 Gm.; calcium carbonate, 1 Gm.; and water, 1000 ml. Due to the surface active property of aspartocin, the fermentation presented an unusual foaming problem, which required large quantities of Hodag oil antifoam. The fermentation was generally harvested after 90 hours of growth at 28 C. Yields in 1000 gallon tanks were generally 4000 to 6000 µg./ml.

ISOLATION AND PROPERTIES

To 1500 liters of fermented mash were added 1500 Gm. of calcium chloride and 45 Kg. of Hyflo Super-Cel diatomaceous earth. The mixture was adjusted to pH 5.0 to 5.5 with concentrated hydrochloric acid, stirred for 20 minutes, and filtered. The filter cake was washed with 300 liters of water, and the wash was discarded. The cake was then suspended in 375 liters of water, pH was adjusted to 9.7 to 10.0 with sodium hydroxide solution, stirred for 20 minutes, and filtered. This extraction procedure was repeated a second time. The combined alkaline extracts of the mash

* These cultures were identified by Drs. E. J. Backus and H. D. Tresner of our laboratories. *S. griseus* var. *spiralis* is Lederle isolate A-8999; *S. violaceus* is Lederle isolate T-3910.

cake were adjusted to *pH* 1.0 to 2.0 with concentrated hydrochloric acid and extracted with 375 liters of *n*-butanol. The butanol extract was adjusted to *pH* 6.0 to 7.0 and concentrated to 15 liters. One hundred fifty Gm. of calcium chloride in 1 liter of water-saturated butanol was added to the butanol concentrate and the *pH* was adjusted between 5.0 and 5.5. The white microcrystalline calcium salt of aspartocin was removed by centrifugation, washed four times with wet butanol and two times with acetone, and air dried. This process resulted in approximately 1200 Gm. of aspartocin calcium salt (75 per cent over-all recovery of activity) of 97 to 100 per cent purity.

For elemental analysis, the aspartocin obtained at its isoelectric point was a more suitable form. The free aspartocin was prepared in amorphous form by dissolving 1 Gm. of calcium salt in 25 ml. of water to which 6 *N* hydrochloric acid was added until a clear solution was obtained at *pH* 1.5. This solution was then adjusted to *pH* 3.3 with 3 *N* sodium hydroxide and to this was added 15 ml. of saturated saline solution. The aspartocin precipitate was removed by centrifugation. After being washed freely with distilled water, the precipitate was dissolved in 50 ml. of methanol, the solution was filtered, and the filtrate was concentrated to approximately 10 ml. On addition of 40 ml. of distilled water, aspartocin was precipitated as an oil, centrifuged, washed with water, triturated with acetone, washed further with acetone, and dried. For analyses, the aspartocin was dried at 100 C. under vacuum over phosphorus pentoxide. Analysis was as follows: C = 53.58, 53.18, 53.31; H = 7.58, 7.58, 7.39; N = 13.58, 13.14; S = 0.36, 0.49; Cl = 0.00, 0.14; ash = 0.82, 0.42; NH₂ (Van Slyke) = 4.27.

The infrared absorption was of the usual nondescript type characteristic of polypeptides. Aspartocin has no characteristic ultraviolet absorption.

$$[\alpha]_D^{25} = +26.4 \text{ degrees (C, 2.1 in methanol).}$$

The crystalline sodium and potassium salts were prepared by the addition of ethanolic solutions of sodium hydroxide or potassium hydroxide to an ethanol solution of aspartocin. The sodium or potassium salts precipitated in microcrystalline form.

Aspartocin is soluble in excess of 50 mg./ml. in methanol, ethanol, and glacial acetic acid. It dissolves slowly in water and *n*-butanol. It is relatively insoluble in acetone and ethyl acetate. The antibiotic is more readily soluble in water at *pH* <3.0 or >3.6. When precipitated at *pH* 3.3, the aspartocin dissolves on the addition of calcium chloride and it can be reprecipitated by sodium chloride.

Aspartocin is relatively stable in aqueous solution. In table I are indicated residual activities of solutions treated under various conditions.

Although aspartocin has chemical and biological properties similar to those of amphotycin, the two antibiotics can be readily separated by paper chromatography or paper electrophoresis. Individual and mixed spots of aspartocin and amphotycin were subjected to descending paper chromatography with 5 per cent ammonium chloride solution as developing agent. The development of the paper chromatogram was terminated after six to seven hours. This is approximately double the time necessary for the solvent to descend to the bottom of the paper strip. Bioautography indicated that aspartocin moved approximately 1 inch, whereas amphotycin trav-

TABLE I

Residual Activities of Solutions under Various Conditions

Samples, pH	% activity remaining after treatment		
	Samples stored 72 hours at 50 C.	Samples stored 30 minutes at	
		70 C.	100 C.
1.0	100	91	37
3.0	91	56	6
5.0	100	79	72
7.0	92	83	57
9.0	100	74	52

eled 6 to 7 inches. Zone electrophoresis on paper wetted with 5 per cent acetic acid showed that amphomycin had approximately six times the mobility of aspartocin toward the cathode.

In vitro assays against *Bacillus subtilis*, *Corynebacterium xerosis*, and *Streptomyces hemolyticus* NY5 indicated aspartocin was four to eight times as active as amphomycin.

Aspartocin gives a positive biuret test and shows rapid uptake of bromine and decolorization of potassium permanganate. Negative reactions were obtained with Millon, xanthoproteic, Sakaguchi, tryptophan (Tillman-Alt), sodium nitroprusside, Molisch, and anthrone tests.

CHEMICAL CONSTITUTION

Aspartocin was hydrolyzed in a sealed tube with 6 *N* hydrochloric acid at 120 C. for 16 hours. The resultant hydrolysate was ninhydrin positive. An oil film was apparent on the surface of the hydrolysate, and it was readily extracted by ether or chloroform. On drying and evaporation, the solvent soluble fraction accounted for 14 to 16 per cent of the weight of aspartocin. This fraction had properties characteristic of unsaturated fatty acids, was acidic, could be esterified with methanolic hydrochloric acid, and readily decolorized bromine.

Two dimensional paper chromatography of the chloroform-extracted hydrolysate indicated seven ninhydrin-positive components when the ninhydrin reaction was conducted at 100 C. The first solvent system used consisted of pyridine-isoamyl alcohol-water (35:30:30), and this was followed with a system consisting of *n*-butanol-formic acid-water (300:53:40).⁶ Bioassays and paper chromatographic mobilities identified four of these components as aspartic acid, glycine, L-proline, and L-valine. Quantitative ninhydrin and microbiological determinations⁷ indicated the contents of these amino acids in aspartocin to be 35, 10, 8, and 8 per cent respectively or in molar ratios of 4:2:1:1. By paper electrophoresis the three remaining ninhydrin-positive components were identified as acidic, neutral, and basic products. The acidic product has R_f values corresponding to those of glutamic acid, but a microbiological assay for glutamic acid was negative.⁷

The presence of L-aspartic acid, L-valine, L-proline, and glycine was confirmed by isolation of these amino acids. The solvent-extracted hydrolysate of aspartocin was chromatographed on sheets of Whatman 3MM paper with a *n*-butanol-acetic acid-water system (4:1:5).⁸ Bands were located by ninhydrin reaction and those corresponding to aspartic acid plus glycine, proline plus the unidentified acidic component, and valine were cut out. All bands were extracted with water.

The extract from the aspartic acid bands was passed through a column of Dowex 50-X8 cation exchange resin (H⁺ form). The column was washed with water and percolated with 5 per cent acetic acid. The acetic acid solution was evaporated to yield crystals, and on recrystallization from water, a white product was obtained that had an infrared spectrum identical with that of L-aspartic acid.

The amino acids from the "proline" band were adsorbed on a Dowex 50-X8 cation exchange resin column and the column was washed with water and 5 per cent acetic acid. The proline was released with 0.2 *N* ammonium hydroxide. This solution was concentrated in vacuo, and on high vacuum sublimation, a white crystalline product was obtained. Infrared analysis confirmed the identity of this product as L-proline.

The "valine" band extracts were likewise passed through a Dowex 50-X8 cation exchange resin column. The column was washed with water and the amino acid was released with 0.2 *N* ammonium hydroxide. Evaporation of this solution and crystallization from aqueous ethanol resulted in a product with a characteristic L-valine infrared spectrum.

Continuous curtain electrophoresis of a hydrolysate in 0.1 *N* acetic acid gave a fraction that on concentration yielded a crystalline material having the infrared spectrum of glycine.

SUMMARY

The production of aspartocin by *S. griseus* var. *spiralis* and *S. violaceus* has been described. Aspartocin is a polypeptide molecule consisting of L-aspartic acid, L-proline, L-valine, glycine, a fatty acid moiety, and three unidentified ninhydrin-positive components.

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Aspartocin. II. Fermentation Studies

MARJORIE A. DARKEN, ALDEN L. JENSEN, AND PING SHU

*Biochemical Research Section, Lederle Laboratories Division,
American Cyanamid Co., Pearl River, N. Y.*

The polypeptide antibiotic aspartocin, which was first reported from these laboratories,¹ is produced by *Streptomyces griseus* var. *spiralis*. This organism has been grown in fermentors ranging from 50 to 1000 gallons, and tank yields of the antibiotic in natural media have varied between 4000 and 8000 $\mu\text{g./ml.}$; in flasks, a potency as high as 12,000 $\mu\text{g./ml.}$ has been observed. The organism has a relatively low oxygen requirement, and phosphate ion markedly interferes with the synthesis of the antibiotic. In order to study the fermentation more closely, simple chemically defined media were also investigated. In these media carbon and nitrogen sources as well as the concentration of monopotassium phosphate were found to be highly critical, and antibiotic yields of 3500 $\mu\text{g./ml.}$ have been obtained in flask fermentations.

EXPERIMENTAL METHODS

Flask Fermentation. Subcultures were carried at 28 C. on yeast malt agar slants, and transfers were made directly to the inoculum flasks containing 100 ml. sterile medium. The inoculated flasks were incubated at 28 C. for 48 hours on a reciprocating shaker with two inch stroke at 104 cycles/minute. Four per cent by volume of the inoculum was added to 250 ml. Erlenmeyer flasks containing 75 ml. of medium. The flasks were then incubated at 28 C. on a rotary Gump shaker operating at 240 r.p.m. In general, fermentations were extended for a seven day period, with daily sampling beginning on the fourth day.

Tank Fermentation. For tank fermentations a two stage vegetative inoculum at a concentration of 0.8 to 4.0 per cent was used. Studies were carried out in 50, 650, and 1000 gallon fermentors. The rate of air flow was 0.6 volume/volume medium/minute at 10 lb./sq. in. gauge over the surface of the medium; no air was passed through the sparger. An agitator speed of 80 to 100 r.p.m. was maintained, and the temperature was kept at 28 C. throughout the 100 hour fermentation period.

Assay. Samples for assay were prepared as previously described¹ and were tested against a strain of *Bacillus subtilis* using the agar plate diffusion method.

FERMENTATION STUDIES IN NATURAL MEDIA

Our medium for the fermentation of aspartocin, as previously disclosed,¹ consists of: protopeptone, 30 Gm.; refined molasses, 20 Gm.; Edamin protein hydrolysate, 5 Gm.; magnesium sulfate, 5 Gm.; calcium carbonate, 1 Gm.; and water, 1000 ml. In the development of this medium, various organic nitrogen sources were investigated in shaker flasks and all were inferior to protopeptone. These nitrogen sources, in decreasing order of productivity, include peptone, Edamin protein hydrolysate, animal stick liquor, oatmeal, barley flour, corn meal, soybean meal, fish meal, wheat flour, cottonseed meal, skimmed milk powder, dried yeast, soy peptone, and corn steep liquor. The addition of nitrate and ammonium nitrogen

TABLE I

Effect of Phosphate on the Biosynthesis of Aspartocin in Shaker Flasks with Natural Medium

Potassium dihydrophosphate concentration, Gm./liter	Phosphate equivalent, Gm./liter	Aspartocin yield, $\mu\text{g./ml.}$
None		4770
0.010	0.007	4470
0.025	0.017	3910
0.050	0.035	3430
0.100	0.070	2900
0.200	0.140	930
0.300	0.210	360
0.400	0.280	35
0.500	0.350	25

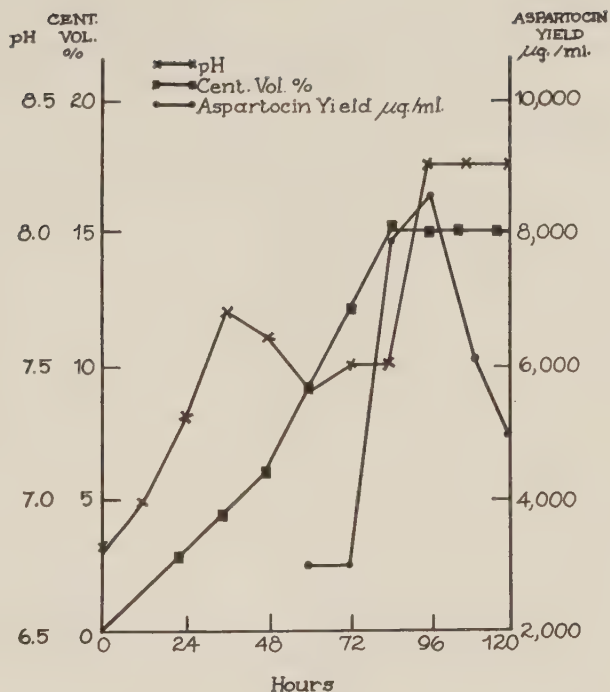
to the medium was without detectable effect on antibiotic production. Several carbon sources were also tested, and none was superior to the combination of corn starch and molasses.

An outstanding characteristic of the fermentation of aspartocin was found to be its great sensitivity to inorganic phosphate. A number of reports have appeared in the literature concerning the significance of phosphorus in the production of antibiotics by various microorganisms.²⁻⁴ Our original inoculum medium⁵ contained 2.7 Gm./liter of added inorganic phosphate, which at 4 per cent carry-over is equivalent to 0.1 Gm./liter in the fermentation medium; this amount seriously impaired antibiotic production, and a yield of 25 $\mu\text{g./ml.}$ was obtained under these conditions. When inorganic phosphate was omitted from the inoculum medium, the aspartocin potency was raised to 4770 $\mu\text{g./ml.}$ The addition of monopotassium phosphate to the fermentation medium clearly demonstrates the toxicity of the phosphate ion, as shown in table I. When the inoculum medium was changed from one containing corn steep liquor (which is relatively high in phosphate) to the aspartocin fermentation medium with protopeptone, yields were further raised to 7100 $\mu\text{g./ml.}$ The effect of phosphate on the assay was subsequently checked; monopotassium phosphate was incorporated in the assay diluent and was found to be without influence on the zone of inhibition. Recovery data from tank fermentations also substantiated this increase in antibiotic potency associated with reduced phosphate concentration.

The influence of phosphate ion on the production of aspartocin was investigated with another soil isolate, *Streptomyces violaceus*. The production of the antibiotic by this culture was also found to be reduced in the presence of added phosphate. In addition, a third culture producing amphomycin⁶ (an antibiotic similar to aspartocin but differing by paper chromatography and paper electrophoresis¹) was studied; this fermentation was also sensitive to added phosphate.

An investigation of air rates was undertaken early in our fermentation studies with *S. griseus* var. *spiralis*. Since the availability of oxygen in shaker flasks is readily altered by changing the volume of medium, fermentations were carried out with volumes ranging from 15 to 75 ml. Significantly greater yields were found at the lower air rate. This response is rare in fermentation studies. With this particular

FIG. 1. Biochemical change pattern in 1000 gallon fermentor with *S. griseus* var. *spiralis* UV75.



organism, the oxygen requirement appears to be unusually low, as sealing of the flasks with Parafilm after a growth period of 24 hours did not diminish antibiotic production. Reduced aeration was also demonstrated to be preferable in tank fermentations.

An ultraviolet irradiated strain, UV75, which was received from M. E. Swift of the Microbiology Department, gave aspartocin yields in shaker flasks as high as 12,000 µg./ml. in contrast to 7000 µg./ml. for the parent culture. In tank fermentation, potencies of 2000 to 3000 µg./ml. have been obtained with the parent strain and 4000 to 8000 µg./ml. with UV75.

In fermentors, the yield of aspartocin reaches a maximum in approximately four days with the recommended medium. Figure 1 presents a biochemical change pattern with strain UV75 in a 1000 gallon fermentor containing 1500 liters of medium. After 95 hours at 28 C. the fermentation was completed with a potency of 8360 µg./ml. The pH starts at neutrality and rises slowly to alkaline levels after a slight dip. There is an active primary growth phase followed by a period of maximum antibiotic production during which the mycelial volume remains essentially constant. The harvest time is critical with an appreciable drop in potency on extended fermentation, but broths are relatively stable under standard harvest conditions.

FERMENTATION STUDIES IN CHEMICALLY DEFINED MEDIA

The methods of fermentation in chemically defined media were the same as those employed for natural media, with the exception that mycelial inoculum growth was centrifuged (10 minutes at 2000 r.p.m.) and washed twice with and finally re-suspended in equivalent amounts of sterile physiological saline before being used at the 1 per cent level for inoculation. The base medium for these nutritional studies

TABLE II

Effect of Phosphate on the Biosynthesis of Aspartocin in Shaker Flasks with Chemically Defined Medium

Potassium dihydrophosphate concentration, Gm./liter	Aspartocin yield, $\mu\text{g./ml.}$
None	15
0.01	215
0.05	495
0.075	700
0.08	800
0.09	1100
0.10	1135
0.11	1120
0.125	1000
0.15	1000
0.20	640
0.40	225
0.50	150
1.00	175

consists of: sodium nitrate, 3.0 Gm.; potassium chloride, 0.5 Gm.; magnesium sulfate, 0.5 Gm.; calcium carbonate, 1.0 Gm.; citric acid, 0.1 Gm.; and deionized water, 1000 ml. The carbon sources were sterilized separately and added prior to inoculation. The strain employed was UV75.

The effect of monopotassium phosphate on the biosynthesis of aspartocin was investigated at levels ranging between 0.01 and 1.0 Gm./liter in the base medium with 25 Gm./liter glucose. A concentration of 0.1 Gm./liter was found to be preferable for the fermentation and was maintained in further studies. The optimum range of monopotassium phosphate was critical and varied from 0.09 to 0.11 Gm./liter, with decreases in yield evident at higher or lower levels. These data are presented in table II.

Magnesium sulfate, potassium chloride, and calcium carbonate were also studied in this medium for their effect on antibiotic production. The levels were not highly critical, and the concentrations used in the base medium were satisfactory for each of these salts. Ferrous sulfate within the limits of 1 to 100 $\mu\text{g./ml.}$ was found to be detrimental to the production of aspartocin. Hoagland's A-Z mineral supplement also was associated with reduced broth potencies when used at a concentration of

TABLE III

Effect of Nitrogen Source on the Biosynthesis of Aspartocin in Shaker Flasks with Chemically Defined Medium

Nitrogen source, 3 Gm./liter	Aspartocin yield, $\mu\text{g./ml.}$
Sodium nitrate	1535
Sodium nitrite	65
Ammonium acetate	720
Ammonium chloride	345
Ammonium lactate	175
Ammonium nitrate	320
Asparagin	240
Urea	655

TABLE IV

Effect of Carbon Source on the Biosynthesis of Aspartocin in Shaker Flasks with Chemically Defined Medium

Carbon source, 25 Gm./liter	Antibiotic yield, $\mu\text{g.}/\text{ml.}$
Glucose	1075
d(+) Galactose	930
d(+) Cellobiose	900
d(+) Maltose	295
d(+) Mannose	185
Glycerol	145
Starch	110
Lactose	100
d(+) Trehalose	100
l(-) Sorbose	80
d(-) Arabinose	75
l(+) Rhamnose	70
d(+) Xylose	70
Butanol	70
Inulin	60
Mannitol	55
Salicin	55
Propanol	55
Sorbitol	50
Adnitol	50
d(+) Melezitose	45
d(+) Melibiose	40
d(+) Raffinose	40
Dulcitol	40
Inositol	40
Methanol	35
Ethanol	35
Sucrose	30
d(-) Melibiose	20
l(+) Arabinose	20
d(-) Levulose	20
Dextran	<10

1 ml./liter. The addition of the amino acids known to be present in aspartocin was without detectable effect on the fermentation yield. Among those used were aspartic acid, glycine, L-proline, and L-valine.

The influence of nitrogen on antibiotic production was investigated; the data, which are presented in table III, clearly demonstrate that the nitrogen source has a great influence on the yield of aspartocin. Nitrate nitrogen as supplied in the base

TABLE V

Chemically Defined Media for the Biosynthesis of Aspartocin

Inoculum	Gm./liter	Fermentation	Gm./liter
Maltose	25	Glucose	25
Sodium nitrate	3	Maltose	10
Potassium chloride	0.5	Sodium nitrate	3
Hydrated magnesium sulfate	0.5	Potassium chloride	0.5
Potassium dihydrophosphate	0.5	Hydrated magnesium sulfate	0.5
Calcium carbonate	1.0	Potassium dihydrophosphate	0.1
Citric acid	0.1	Calcium carbonate	1.0
Water	1000	Citric acid	0.1
		Water	1000

medium was found to be superior to nitrite, ammonia, urea, or asparagin. These other nitrogen sources were tested at a level of 3 Gm./liter in a medium to which sodium chloride had been added to provide the sodium ion normally supplied by sodium nitrate.

Thirty-two carbon sources were tested at a concentration of 25 Gm./liter in the base medium with 0.1 Gm./liter monopotassium phosphate. The results are presented in table IV. Glucose was superior to all other carbon sources investigated for maximum antibiotic production, (d+) cellobiose and (d+) galactose were slightly inferior, and the other carbon sources were associated with considerably reduced antibiotic formation. Maltose supported extremely heavy growth although yields were only 25 per cent of those obtained with glucose. Maltose was therefore tried as an adjuvant to medium containing glucose. The aspartocin potency in synthetic media was raised from 1075 to 3000 µg./ml., and mycelial growth was substantially greater when maltose was added to the fermentation medium at a concentration of 10 Gm./liter.

It is of interest to note that with maltose as the sole carbon source in a chemically defined inoculum medium having the proper phosphate concentration, yields in the synthetic fermentation medium were further increased to 3500 µg./ml.

The formulas for the inoculum and fermentation media recommended for the production of aspartocin under chemically defined conditions are given in table V. These media differ not only in carbon source but also in phosphate concentration.

SUMMARY

An investigation of the nutritional requirements of *S. griseus* var. *spiralis* for the production of aspartocin in both natural and chemically defined media has been described. The organism has a relatively low oxygen requirement, and phosphate ion markedly interferes with the synthesis of the antibiotic. The importance of preferential carbon and nitrogen sources, as well as of other required nutrients, is discussed. Laboratory fermentations were readily adapted to tank scale.

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Aspartocin. III. In Vitro Antimicrobial Properties

E. J. KIRSCH, A. C. DORNBUSH, AND E. J. BACKUS

*Biochemical Research Section, Lederle Laboratories,
American Cyanamid Co., Pearl River, N. Y.*

Aspartocin is a new biologically active peptide produced by *Streptomyces griseus* var. *spiralis*. This organism is similar to *S. griseus* as described by Waksman with the major exception that the sporophores are spiraled rather than flexuous.¹ The isolation and characterization of the antibiotic are described in a report by Shay et al² and its in vivo properties by Redin and McCoy.⁴

This paper is a compilation of the antimicrobial properties of the drug. Among the topics to be discussed here are: the antimicrobial spectrum, effect of culture environment on antibiotic activity, preliminary investigations of the mode of action, rapidity of resistance development, and cross resistance.

METHODS AND MATERIALS

Antimicrobial Spectrum. The minimal inhibitory concentration of aspartocin was determined by the serial broth dilution assay technique. Except where otherwise noted, Trypticase soy broth was used as the assay medium. In order to facilitate studies on the effects of various nutrients, the medium was usually prepared from the individual components.

Inoculum was prepared by growing the culture for 18 to 24 hours in Trypticase soy broth. The population density was determined at 660 m μ on a Coleman Jr. spectrophotometer and was adjusted to 2×10^6 cells/ml. in 0.9 per cent saline. One drop of the diluted suspension, approximately 1×10^5 cells, was added to each assay tube. The tubes were examined visually after 24 and 48 hours of incubation. The minimal inhibitory concentration was defined as the lowest concentration of antibiotic preventing the appearance of visible growth after 48 hours of incubation at 37 C.

Resistant Strain Development. The development of resistance to aspartocin by two strains of streptococci and two strains of staphylococci was studied in Trypticase soy broth. Transfers were made every 48 hours from the tube containing the highest concentration of antibiotic permitting growth into broth tubes containing this level and several higher concentrations of the antibiotic. Development of resistance to erythromycin was determined concurrently by the same technique.

Mode of Action Studies. The viability of *Staphylococcus aureus* Smith incubated at 37 C. in the presence of various concentrations of aspartocin in Trypticase soy broth was determined by the plate count technique. An 18 hour culture of the organism was diluted in sterile deionized water and added to the broth so that the final viable cell count was in the order of 1.6×10^5 cells/ml. The antibiotic was sterilized by filtration and added to give concentrations of 50.0, 25.0, 12.5, and 6.2 μ g./ml. Aliquots were withdrawn from each tube prior to incubation and at intervals of 3, 6, 24, and 48 hours during incubation at 37 C. The samples were diluted at least tenfold before plating to reduce the antibiotic level below the

TABLE I
Antibacterial Spectrum of Aspartocin

Test organism	Description	Trypticase soy broth	
		MIC* ($\mu\text{g./ml.}$)	Minus inorganic phosphate, MIC ($\mu\text{g./ml.}$)
<i>Staphylococcus aureus</i>	ATCC 6538	15.5	2.0
<i>Staphylococcus aureus</i>	#3 Type I	15.5	2.0
<i>Staphylococcus aureus</i>	(Tetracycline-resistant isolate)	8.0	2.0
<i>Staphylococcus aureus</i>	209P	15.5	2.0
<i>Staphylococcus aureus</i>	(209P spiramycin resistant)	15.5	2.0
<i>Staphylococcus aureus</i>	(209P carbomycin resistant)	15.5	
<i>Staphylococcus aureus</i>	(209P erythromycin resistant)	15.5	1.0
<i>Staphylococcus aureus</i>	(209P leucomycin resistant)	8.0	2.0
<i>Staphylococcus aureus</i>	Smith	15.5	1.0
<i>Streptococcus pyogenes</i>	C203	2.0	0.03
<i>Streptococcus pyogenes</i>	NY5	4.0	0.5
<i>Streptococcus pyogenes</i>	(NY5 spiramycin resistant)	4.0	0.5
<i>Streptococcus pyogenes</i>	(Tetracycline-resistant isolate)	8.0	1.0
<i>Streptococcus pyogenes</i>	(Tetracycline-resistant isolate)	4.0	2.0
<i>Corynebacterium xerosis</i>	NRRL B-1397	1.0	0.12
<i>Bacillus cereus</i>		4.0	4.0
<i>Sarcina lutea</i>		2.0	2.0
<i>Bacillus polymyxa</i>		4.0	2.0
<i>Bacillus megatherium</i>		0.25	0.12
<i>Erysipelothrix rhusopathiae</i>		0.5	
<i>Bacillus subtilis</i>	ATCC 6633	4.0	
<i>Bacillus subtilis</i>	(ATCC 6633 puromycin resistant)	1.0	
<i>Bacillus subtilis</i>	#17	2.0	
<i>Bacillus subtilis</i>	(#17 carbomycin resistant)	2.0	
<i>Klebsiella pneumoniae</i>	"A" strain AD	>250	
<i>Pasteurella multocida</i>	#652	>250	
<i>Salmonella gallinarum</i>	#605	>250	
<i>Escherichia coli</i>		>250	
<i>Proteus vulgaris</i>		>250	
<i>Candida albicans</i>	CA-300	>250	
<i>Mycobacterium ranae</i>		62.0	
<i>Mycobacterium 607</i>		62.0	

* Minimal inhibitory concentration.

minimal inhibitory concentration. Platings were made in duplicate at several dilutions to ensure meaningful counting levels. Plate counts were made after 48 hours' incubation at 37 C. and the values were averaged.

Hemolysis of Sheep Erythrocytes. Tetracycline,* penicillin, tyrothricin, and aspartocin were serially diluted in 0.9 per cent saline. Erythrocytes from defibrinated sheep blood were packed by centrifugation. The serum was removed and replaced by an equal volume of physiological saline. The cell suspension was diluted to 10 per cent and two drops were added to each assay tube. Readings were made after 18 hours of incubation at 37 C. The complete disintegration of the erythrocyte pellet was arbitrarily regarded as a positive hemolytic reaction.

RESULTS AND DISCUSSION

The antibacterial spectrum of aspartocin is shown in table I. The antibiotic was

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for tetracycline is Achromycin.

TABLE II

Distribution of Sensitivity of Clinical Isolates Tested Against Aspartocin, Penicillin, and Chlortetracycline

Antibiotic	Hours of incubation	Per cent staphylococcal isolates at MIC range, $\mu\text{g./ml.}$			Per cent streptococcal isolates at MIC range, $\mu\text{g./ml.}$		
		>25.0	6.0 to 25.0	<6.0	>25.0	6.0 to 25.0	<6.0
Aspartocin	24	0	96	4	0	60	40
	48	4	96	0	0	65	35
Penicillin	24	88	4	8	0	30	70
	48	88	4	8	0	47	53
Chlortetracycline	24	72	4	24	35	12	53
	48	72	24	4	47	41	12

effective against gram-positive bacteria but not against gram-negative bacteria. Some slight activity against the mycobacteria has also been observed.

Included among the test organisms were a number of strains made resistant to some of the better-known antibiotics. Aspartocin inhibited these resistant organisms at approximately the same level found inhibitory for the sensitive parent cultures. Three tetracycline-resistant isolates, for which parent cultures were not available, were also inhibited at an antibiotic concentration close to that observed for related organisms. Although not shown in table I, one example of cross resistance was observed when a culture of *Staph. aureus* Smith made resistant to aspartocin was tested against amphotycin. It was found that this strain was not susceptible to 250 $\mu\text{g./ml.}$ of either amphotycin or aspartocin.

Two sets of minimal inhibitory concentration values are listed in table I. The first column shows the value obtained in complete Trypticase soy broth, while the second set of values was obtained when inorganic phosphate was omitted from the medium. The effect of inorganic phosphate was investigated subsequent to the finding of Darken et al³ of the pronounced effect of this ion on the fermentation of aspartocin. The results indicate that omission of inorganic phosphate from Trypticase soy broth reduces the tolerance of selected microorganisms to the lethal effects of the antibiotic.

The data presented in table II indicate that aspartocin is an effective inhibitor of clinical isolates of staphylococci and streptococci. The sensitivities to aspartocin, penicillin, and chlortetracycline* of 25 cultures of staphylococci and 17 cultures of streptococci isolated from hospitalized patients were determined by the usual tube dilution technique. The cultures were originally selected for their relative insensitivity to penicillin or chlortetracycline and therefore are not to be construed as a random sampling of hospital strains. The data would seem to imply that aspartocin is active against these pathogens over a concentration range approximating the minimal inhibitory concentration of laboratory maintained staphylococci and streptococci.

The rapidity and degree of resistance to aspartocin developed by *Staph. aureus*

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin.

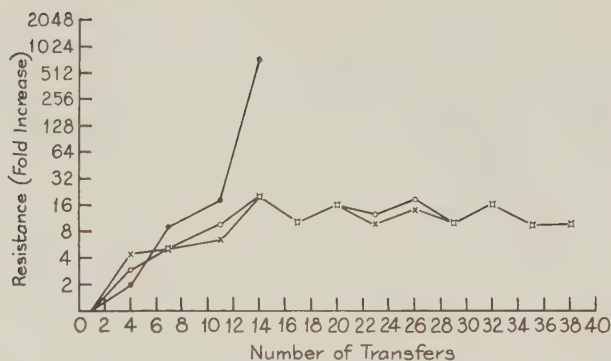


FIG. 1. The pattern of resistance developed by streptococci to aspartocin compared with erythromycin is shown. —•—, *Str. pyogenes* C-203 vs. erythromycin; o—o, *Str. pyogenes* C-203 vs. aspartocin; x—x, *Str. pyogenes* NY-5 vs. aspartocin.

209P, *Staph. aureus* Smith, *Streptococcus pyogenes* NY5, and *Str. pyogenes* C203 are shown in figures 1 and 2. The two staphylococci tested showed an early low-grade rise in resistance but the rate of increase diminished between the sixth and twenty-sixth transfers. *Staph. aureus* 209P remained about 30 times resistant through 40 transfers, but *Staph. aureus* Smith increased in resistance by a factor of 200-fold. In comparison, erythromycin resistance developed in a steady, progressive fashion, so that at the fourteenth transfer, *Staph. aureus* 209P was resistant by a factor of 600-fold. The streptococci tested never developed higher than twentyfold resistance to aspartocin through the entire transfer series.

The viability of *Staph. aureus* Smith, determined by plate counts after incubation in Trypticase soy broth with various concentrations of aspartocin, is presented in figure 3. The logarithm of the viable count is plotted against time of exposure to the antibiotic. The curve, constructed from data obtained at a concentration of 25.0 $\mu\text{g./ml.}$ of antibiotic, clearly demonstrates the bactericidal action of the drug. The rapid decrease in viability with no apparent recovery even after 48 hours' incubation would appear to indicate complete sterilization of the culture. There was evidence of considerable killing at the 12.5 $\mu\text{g./ml.}$ level, but the culture recovered partially after prolonged incubation. Even at the lowest level tested, 6.2 $\mu\text{g./ml.}$, the viable count decreased sharply, indicating the bactericidal nature of the drug. The persistence of uninhibited cells at this level was sufficient to permit rapid proliferation with the result that maximal growth was attained in 24 hours.

A paradoxical situation was observed when the effect of normal horse serum on aspartocin activity was studied. It can be seen in table III that in Trypticase soy broth the organism is protected by the addition of serum, whereas in brain-heart

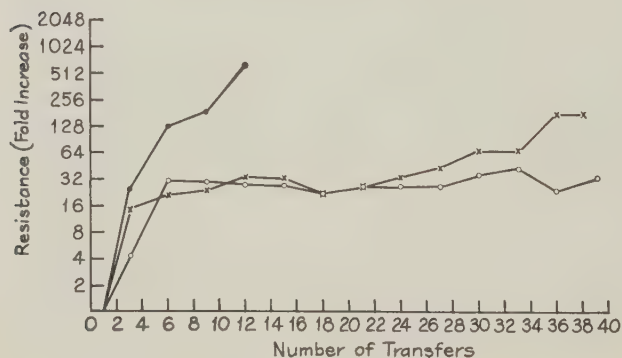
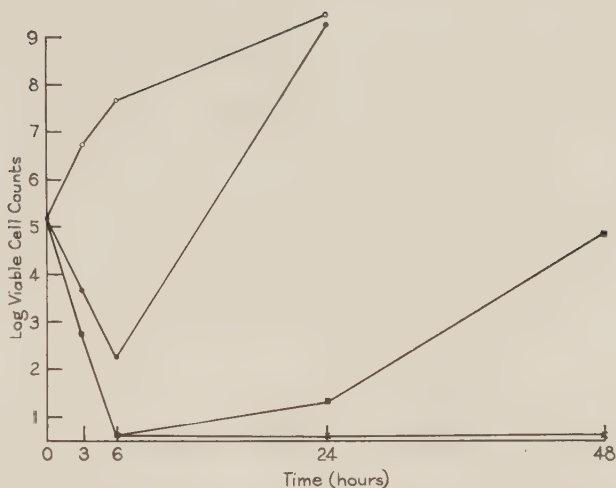


FIG. 2. The pattern of resistance developed by staphylococci to aspartocin compared with erythromycin is illustrated. —•—, *Staph. aureus* 209P vs. erythromycin; o—o, *Staph. aureus* 209P vs. aspartocin; x—x, *Staph. aureus* Smith vs. aspartocin.

FIG. 3. The viability of *Staph. aureus* Smith in various concentrations of aspartocin is shown. o—o, no antibiotic; —, 6.25 µg./ml.; ■—■, 12.5 µg./ml.; x—x, 25.0 µg./ml.



infusion broth the activity of the antibiotic is slightly potentiated by serum. At present no reasonable explanation can be offered for this anomaly.

The effects of culture environment on antistaphylococcal activity of aspartocin are presented in table IV. Considerable variation in activity was obtained when the antibiotic was tested in several different media. Nutrient broth seemed least antagonistic to the action of the drug. Aspartocin activity was not reduced by supplementation of nutrient broth with any one of the complex organic ingredients found in the more antagonistic media. A sixteenfold reversal of aspartocin activity was noted, however, when inorganic phosphate was added to nutrient broth. This result is in direct agreement with the prior observation that aspartocin activity is enhanced by the deletion of inorganic phosphate from the Trypticase soy broth formula. The influence of phosphate on activity was also demonstrated in a synthetic broth medium described by Boniece.⁵ Under these conditions, omission of phosphate effected a fourfold increase in activity.

Potentiation of aspartocin activity was most strikingly observed when 0.09 M

TABLE III
Effect of Normal Horse Serum on the Antibacterial
Activity of Aspartocin

Test organism	Medium	Serum added, %	MIC, µg./ml.
<i>Staph. aureus</i> Smith	TSB*	0	31.0
<i>Staph. aureus</i> Smith	TSB	25	125.0
<i>Staph. aureus</i> Smith	TSB	50	250.0
<i>Staph. aureus</i> Smith	TSB	75	>250.0
<i>Staph. aureus</i> Smith	BHI†	0	125.0
<i>Staph. aureus</i> Smith	BHI	50	31.0
<i>Str. pyogenes</i> C-203	TSB	0	3.0
<i>Str. pyogenes</i> C-203	TSB	25	12.5
<i>Str. pyogenes</i> C-203	TSB	50	25.0
<i>Str. pyogenes</i> C-203	TSB	75	>50.0

*Trypticase soy broth (BBL).

†Brain-heart infusion broth (Difco).

TABLE IV

Effect of Culture Medium on Antistaphylococcal Activity of Aspartocin

Medium	MIC, $\mu\text{g.}/\text{ml.}$
Brain-heart infusion broth	62.5 to 125.0
Trypticase soy broth	15.5 to 31.0
AC broth (Difco)	125.0
Penassay broth (Difco)	125.0
Tryptose phosphate broth (Difco)	125.0
Nutrient broth (Difco)	1.0 to 2.0
Nutrient broth plus 2.0% proteose peptone	2.0
Nutrient broth plus 0.5% glucose	1.0
Nutrient broth plus 0.5% yeast extract	2.0
Nutrient broth plus 0.5% beef extract	1.0
Nutrient broth plus 3.4% Trypticase	2.0
Nutrient broth plus 1.0% casitone	2.0
Nutrient broth plus 0.6% phytone	2.0
Nutrient broth plus 2.0% inorganic phosphate	31.0
Synthetic medium ⁶	62.5
Synthetic medium ⁵ minus inorganic phosphate	16.0
Synthetic medium ⁵ minus inorganic phosphate plus 0.02 M calcium chloride	0.25

calcium chloride was added to brain-heart infusion broth. The antibacterial activity against *Staph. aureus* Smith was increased by a factor of 250-fold. These data are presented in table V, where it is also shown that none of several other metallic cations tested appeared to have any pronounced effect on the activity of the antibiotic. It is well known that the addition of calcium chloride to phosphate-containing medium results in rapid complexing of the phosphate and its precipitation from solution. The results presented therefore suggest that calcium enhances aspartocin activity by reducing the concentration of available phosphate to a level that no longer permits the observation of phosphate antagonism. However, referring again to table IV, it should be noted that the addition of calcium chloride to synthetic broth⁵ containing no inorganic phosphate in its formula permits a 16 times greater enhancement of aspartocin activity than that obtained by omission of phosphate alone. These data would seem to imply that potentiation by calcium in broth media

TABLE V

Effect of Various Cations on Antistaphylococcal Activity of Aspartocin in Brain-Heart Infusion Broth

Compound tested	Molar concentration	
	0.045 M MIC, $\mu\text{g.}/\text{ml.}$	0.09 M MIC, $\mu\text{g.}/\text{ml.}$
No additive	125.0	125.0
Barium sulfate	62.5	125.0
Calcium chloride	16.0	0.5
Hydrated ferric chloride	125.0	125.0
Hydrated iron sulfate	125.0	Toxic
Hydrated magnesium chloride	62.5	125.0
Hydrated manganese chloride	62.5	Toxic
Hydrated zinc sulfate	Toxic	Toxic
Hydrated aluminum sulfate	62.5	125.0
Potassium chloride	125.0	125.0

TABLE VI
Effect of Aspartocin on Sheep Erythrocytes

Antibiotic	Hemolysis of sheep red blood cells, concentration of antibiotic, $\mu\text{g./ml.}$					
	0	0.25	62.0	125.0	250	500
Aspartocin	—	—	—	—	—	+
Tyrothricin	—	+	+	+	+	+
Penicillin	—	—	—	—	—	—
Tetracycline	—	—	—	—	—	+

is probably the result of two processes, the first being the reversal of phosphate antagonism by removal of available phosphate and the second, a direct interaction of calcium, antibiotic, and cell by a mechanism as yet unexplained. Conceivably the latter process could be involved with the absorption of antibiotic at the cell surface.

Table VI presents data indicating that aspartocin does not markedly induce lysis of sheep erythrocytes suspended in saline. Tyrothricin, well known for its hemolytic properties, was active in this respect at a concentration as low as 0.25 $\mu\text{g./ml.}$ Tetracycline and aspartocin were hemolytic only at the highest concentration tested, 500 $\mu\text{g./ml.}$ Penicillin was nonhemolytic at this concentration.

SUMMARY AND CONCLUSIONS

The in vitro antimicrobial properties of the antibiotic aspartocin have been described. The antimicrobial spectrum indicates that the drug is primarily active against gram-positive bacteria. Cultures of penicillin or tetracycline-resistant staphylococci and streptococci isolated from clinical sources are susceptible to the antibiotic. Staphylococci and streptococci do not easily develop high resistance to the drug. Cross resistance is observed between aspartocin and amphotycin but not between aspartocin and any of the several other antibiotics tested.

Variation in the in vitro activity in broth culture appears to be directly related to the nature of the culture environment. Particularly striking is the potentiation of antistaphylococcal activity after either the addition of calcium ions to the medium or the omission of phosphate ions from the medium. The action of the drug on staphylococci appears to be bactericidal at concentrations bordering the minimal inhibitory concentration.

Lysis of sheep erythrocytes was observed only at the highest level of the antibiotic tested, 500 $\mu\text{g./ml.}$ Normal horse serum was shown to have an effect on antibiotic activity, potentiation or antagonism being related to the culture medium in which the test is performed.

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Aspartocin. IV. Activity Against Experimental Infections in Mice

G. S. REDIN AND M. E. MCCOY

Bacterial Chemotherapy Department, Experimental Therapeutics Research, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

A new antibiotic, aspartocin, is produced by *Streptomyces griseus* var. *spiralis*. Studies on the methods of production, isolation, and in vitro antimicrobial properties have been reported.¹⁻³ Aspartocin has been described as a polypeptide active in vitro against various gram-positive bacteria. The available information as to chemistry,¹ activity,³ and toxicity⁴ indicates that aspartocin is similar to amphotycin.⁵ The present report is concerned with the relative potencies of aspartocin and chlortetracycline or tetracycline in four standardized bacterial infections in mice. Preliminary acute toxicity data in mice are also presented.

PRELIMINARY STUDIES

In routine tests to determine activity, aspartocin was inactive at relatively high dosages in the following infections by the routes indicated: *Klebsiella pneumoniae* AD in mice: subcutaneously, intraperitoneally, single oral tubing, and in the drug diet; *Pasteurella multocida* 310 in mice: subcutaneously; *Mycobacterium tuberculosis* H₃₇Rv in mice: subcutaneously; Columbia SK virus in mice: intraperitoneally; pleuropneumonia-like organisms in embryonated eggs: allantoic cavity; *Salmonella gallinarum* 605 in chicks: intraperitoneally.

EXPERIMENTAL STUDIES

Mice. The unit test group of animals consisted of 10 Carworth Farms (CF no. 1 strain) mice, females, 4 to 6 weeks of age. Initial weights averaged 16 to 20 Gm./mouse.

Infections. Infections were produced by intraperitoneal injection of 0.5 ml. volumes of dilutions in Trypticase soy broth of five hour blood broth cultures of each of the following strains: *Streptococcus pyogenes*, beta-hemolytic strain C203: 10⁻⁵ dilution containing 6000 \pm 3000 viable units, as determined by plate counts—the mortality rate for infected untreated controls was 100 per cent of 160 mice, with average survival times ranging from 1.0 to 1.3 days; *Diplococcus pneumoniae*, type I, strain SV1: 10⁻⁶ dilution containing 1500 \pm 500 viable units, as determined by plate counts—the mortality rate for infected untreated controls was 99.5 per cent (179/180), with average survival times ranging from 2.0 to 2.1 days; *Staphylococcus aureus*, strain Smith: 10⁻² dilution containing 16 \times 10⁶ \pm 6 \times 10⁶ viable units, as determined by plate counts—the mortality rate for infected untreated controls was 99.4 per cent (159/160), with average survival times ranging from 1.0 to 1.4 days; *Staph. aureus*, strain Rose (tetracycline resistant): undiluted culture containing 8 \times 10⁸ \pm 2 \times 10⁸ viable units, as determined by plate counts—the mortality rate for infected untreated controls was 99.2 per cent (119/120), with average survival times ranging from 1.0 to 1.1 days.

Antibiotic Preparations. The experiments involving intraperitoneal and subcutaneous treatment in the *Str. pyogenes* C203, *D. pneumoniae* SV1, and *Staph. aureus* Smith infections were run at an early stage in the development of aspartocin, and the aspartocin used was relatively crude material. Later, the sodium salt was made from a preparation of aspartocin about twice as pure as the earlier material, as measured by in vitro assay, and this was used for the tetracycline-resistant *Staph. aureus* Rose experiment (table II), the intravenous treatment experiments (table IV), and the acute toxicity work (table VIII).

Treatment. Treatment was administered by single subcutaneous, intraperitoneal, or intravenous injection. For subcutaneous or intraperitoneal treatment, the antibiotics were dissolved in 0.2 per cent aqueous agar and were injected in 0.5 ml. volumes immediately after infection (total infecting time was about one-half hour). Volumes of 0.2 ml. (aqueous solutions) were administered intravenously. For each test, each drug was tested at four to six different dosages, covering an 8- to 32-fold

TABLE I
*Activity of Aspartocin and Chlortetracycline Administered by Single Subcutaneous Injection
in Standardized Injections in Mice*

Infection	Dose, mg./Kg.	Survival on the fourteenth day after infection			
		Chlortetracycline		Aspartocin	
		Alive/ total	Per cent effect	Alive/ total	Per cent effect
<i>Streptococcus pyogenes</i> C203	40	20/20	100	—	—
	20	29/40	73	40/40	100
	10	27/40	68	37/40	93
	5	18/40	45	36/40	90
	2.5	9/30	30	1/40	3
	1.25	0/20	0	0/40	0
Median effective dose	5.8 (4.2–8.1) mg./Kg.		5.0 (2.0–13) mg./Kg.		
<i>Diplococcus pneumoniae</i> SV1	40	40/40	100	—	—
	20	25/40	63	—	—
	10	3/40	8	40/40	100
	5	3/40	8	40/40	100
	2.5	0/30	0	33/40	83
	1.25	—	—	0/40	0
Median effective dose	17 (10–28) mg./Kg.		2.0 (1.8–2.3) mg./Kg.		
<i>Staphylococcus aureus</i> Smith	10	—	—	40/40	100
	5	—	—	12/40	30
	2.5	39/40	98	2/40	5
	1.25	26/40	65	1/40	3
	0.62	10/40	25	—	—
	0.31	4/30	13	—	—
	0.15	0/30	0	—	—
Median effective dose	0.8 (0.7–1.0) mg./Kg.		5.5 (2.6–12) mg./Kg.		
<i>Control Mice</i>					
<i>Streptococcus pyogenes</i> C203:		80 of 80 infected untreated mice died with average survival times of 1.0 to 1.3 days			
<i>Diplococcus pneumoniae</i> SV1:		79 of 80 infected untreated mice died with an average survival time of 2.0 days			
<i>Staphylococcus aureus</i> Smith:		79 of 80 infected untreated mice died with average survival times of 1.0 to 1.1 days			
Forty-eight of 50 uninfected untreated mice were alive on the fourteenth day after infection, at which time the tests were terminated					

TABLE II

Activity of Aspartocin (Sodium Salt) Administered by Single Subcutaneous Injection in a Tetracycline-Resistant Staphylococcus aureus Rose Infection in Mice

Infection	Dose, mg./Kg.	Survival on the fourteenth day after infection	
		Alive/total	Per cent effect
<i>Staphylococcus aureus</i> Rose	128	26/30	87
	64	26/30	87
	32	6/30	20
	16	1/30	3
Median effective dose		39 (33-48) mg./Kg.	

Chlortetracycline was ineffective at a dosage of 1024 mg./Kg.

Sixty of 60 infected untreated control mice died with average survival times of 1.0 to 1.1 days.

TABLE III

Activity of Aspartocin and Chlortetracycline Administered by Single Intraperitoneal Injection in Standardized Infections in Mice

Infection	Dose, mg./Kg.	Survival on the fourteenth day after infection			
		Chlortetracycline		Aspartocin	
		Alive/ total	Per cent effect	Alive/ total	Per cent effect
<i>Streptococcus pyogenes</i> C203	160	10/10	100	—	—
	80	28/30	93	—	—
	40	24/40	60	—	—
	20	10/40	25	—	—
	10	0/40	0	—	—
	5	0/10	0	40/40	100
	2.5	—	—	34/40	85
	1.25	—	—	8/40	20
	0.62	—	—	0/30	0
Median effective dose		33 (27-40) mg./Kg.		1.7 (1.5-2.0) mg./Kg.	
<i>Diplococcus pneumoniae</i> SV1	20	38/40	95	—	—
	10	26/40	65	—	—
	5	1/40	3	—	—
	2.5	0/40	0	20/20	100
	1.25	0/40	0	18/20	90
	0.62	—	—	23/40	58
	0.31	—	—	6/40	15
	0.16	—	—	3/40	8
	0.08	—	—	0/20	0
Median effective dose		9.7 (8.0-12) mg./Kg.		0.6 (0.5-0.7) mg./Kg.	
<i>Staphylococcus aureus</i> Smith	2.5	40/40	100	39/40	98
	1.25	35/40	88	37/40	93
	0.62	23/40	58	22/40	55
	0.31	16/40	40	7/40	18
	0.16	5/40	13	0/10	0
Median effective dose		0.5 (0.4-0.6) mg./Kg.		0.7 (0.5-0.8) mg./Kg.	

Control Mice

Streptococcus pyogenes C203: 60 of 60 infected untreated mice died with an average survival time of 1.0 day

Diplococcus pneumoniae SV1: 80 of 80 infected untreated mice died with average survival times of 2.0 to 2.1 days

Staphylococcus aureus Smith: 60 of 60 infected untreated mice died with average survival times of 1.1 to 1.4 days

Forty of 40 uninfected untreated mice were alive on the fourteenth day after infection, at which time the tests were terminated

TABLE IV

Activity of Aspartocin (Sodium Salt) and Tetracycline Administered by Single Intravenous Injection Against the Staphylococcus aureus Smith (Tetracycline Sensitive) and the Staphylococcus aureus Rose (Tetracycline Resistant) Infections in Mice

Dose, mg./Kg.	Survival on fourteenth day postinfection <i>Staphylococcus aureus</i> Smith				Survival on seventh day postinfection <i>Staphylococcus aureus</i> Rose			
	Aspartocin		Tetracycline		Aspartocin		Tetracycline	
	Alive/ total	% effect	Alive/ total	% effect	Alive/ total	% effect	Alive/ total	% effect
64*	—	—	—	—	20/20	100	1/30	3
32	—	—	—	—	30/30	100	0/10	0
16	—	—	—	—	24/30	80	1/10	10
8	—	—	—	—	1/30	3	0/10	0
4	30/30	100	30/30	100	1/30	3	—	—
2	29/30	97	25/30	83	—	—	—	—
1	13/30	43	14/30	47	—	—	—	—
0.5	6/30	20	11/30	37	—	—	—	—
0.25	1/30	3	3/30	10	—	—	—	—
<i>Infected controls:</i> 96.7% (58 of 60) infected untreated control mice died with average survival times of 1.0 to 1.2 days					<i>Infected controls:</i> 98.3% (59 of 60) infected untreated control mice died with average survival times of 1.0 day			
<i>Uninfected controls (0.2 ml. water):</i> 20 of 20 uninfected vehicle control mice were alive on fourteenth day when test was terminated					<i>Uninfected controls (0.2 ml. water):</i> 30 of 30 uninfected vehicle control mice were alive on seventh day when test was terminated			

* Maximum tolerated single intravenous dose for tetracycline.

TABLE V

Dose-Effect Curve Parameters of Aspartocin and Chlortetracycline Administered by Single Subcutaneous Injection in Standardized Infections in Mice

Antibiotic	Median effective dose, mg./Kg.	Slope function*	Relative activity
<i>Streptococcus pyogenes</i> C203			
Chlortetracycline	5.8 (4.2–8.1)	4.8 (2.9–8.1)	1.00
Aspartocin	5.0 (2.0–13)	1.6 (0.96–2.6)	1.2†
<i>Staphylococcus aureus</i> Smith			
Chlortetracycline	0.8 (0.7–1.0)	1.8 (1.6–2.1)	1.00
Aspartocin	5.5 (2.6–12)	1.8 (1.2–2.9)	0.15 (0.06–0.34)
<i>Diplococcus pneumoniae</i> SV1			
Chlortetracycline	17 (10–28)	1.9 (1.3–2.7)	1.00
Aspartocin	2.0 (1.8–2.3)	1.3 (1.2–1.4)	8.3 (4.8–14)
<i>Staphylococcus aureus</i> Rose			
Chlortetracycline	inactive at 1024	—	—
Aspartocin	39 (33–48)	1.5 (1.3–1.7)	—

Figures in parentheses indicate 95 per cent confidence limits.

* Slope function = $\frac{ED_{84}/ED_{50} + ED_{60}/ED_{15}}{2}$

† Dose-response curve deviate significantly from parallelism.

TABLE VI

Dose-Effect Curve Parameters of Aspartocin and Chlortetracycline Administered by Single Intraperitoneal Injection in Standardized Infections in Mice

Antibiotic	Median effective dose, mg./Kg.	Slope function*	Relative activity
<i>Streptococcus pyogenes</i> C203			
Chlortetracycline	33 (27-40)	1.9 (1.6-2.2)	1.00
Aspartocin	1.7 (1.5-2.0)	1.4 (1.3-1.6)	19†
<i>Staphylococcus aureus</i> Smith			
Chlortetracycline	0.5 (0.4-0.6)	2.2 (1.8-2.8)	1.00
Aspartocin	0.7 (0.5-0.8)	1.9 (1.5-2.1)	0.7 (0.6-0.9)
<i>Diplococcus pneumoniae</i> SV1			
Chlortetracycline	9.7 (8.0-12)	1.6 (1.4-1.8)	1.00
Aspartocin	0.6 (0.5-0.7)	1.9 (1.7-2.2)	16†

Figures in parentheses indicate 95 per cent confidence limits.

* Slope function = $\frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$.

† Dose-response curves deviated significantly from parallelism.

range on a twofold scale with 10 mice at each dosage. The dosages were adjusted after preliminary testing in an attempt to obtain survival ratios ranging from 0 to 100 per cent.

The effect of orally administered aspartocin on the *Str. pyogenes* C203, *D. pneumoniae* SV1, and *Staph. aureus* Smith infections was tested by either a single oral tubing dose at the time of infection (320 mg./Kg.) or in the diet at a level of 0.4 per cent starting one day prior to infection (approximately 500 mg./Kg./day of antibiotic).

Evaluation of Therapeutic Activity. The quantitative effectiveness of each antibiotic was measured by means of Litchfield and Wilcoxon's method of evaluating dose-effect experiments.⁶ The activity of each antibiotic in each infection was determined in terms of the median effective dose (ED_{50}) in mg./Kg. The relative potency of aspartocin was obtained by dividing its ED_{50} into the corresponding value for chlortetracycline or tetracycline. In 3 cases the curves were not parallel, and the potency ratio limits were not calculated.

TABLE VII

Dose-Effect Curve Parameters of Aspartocin and Tetracycline Administered by Single Intravenous Injection Against the Staphylococcus aureus Smith and Staphylococcus aureus Rose Infections in Mice

Drug	Median effective dose, mg./Kg.	Slope function*	Relative activity
<i>Staphylococcus aureus</i> Smith			
Tetracycline	0.82 (0.63-1.1)	2.1 (1.7-2.6)	1.00
Aspartocin	0.94 (0.70-1.3)	1.8 (1.1-3.1)	0.87 (0.58-1.3)
<i>Staphylococcus aureus</i> Rose			
Tetracycline	Inactive at 64	—	—
Aspartocin	13 (11-15)	1.4 (1.2-(1.5)	>5

Figures in parentheses indicate 95 per cent confidence limits.

* Slope function = $\frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$.

TABLE VIII

Acute Toxicity of Aspartocin (Sodium Salt) and Tetracycline in Mice

Dose, mg./Kg.	Dead/total mice tested, 6 days after dosing	
	Aspartocin	Tetracycline
<i>Intraperitoneally</i>		
512	—	17/20
256	20/20	1/20
128	14/20	0/20
64	1/20	—
Median lethal dose	110 (88–130)	380 (estimate)
<i>Subcutaneously</i>		
1024	—	29/30
512	20/20	16/29
256	26/30	1/30
128	0/30	—
Median lethal dose	200 (estimate)	500 (410–600)
<i>Intravenously</i>		
512	—	39/40
256	39/40	29/40
128	24/40	6/40
64	0/40	—
Median lethal dose	120 (96–140)	200 (170–240)

RESULTS

Results of replicate evaluation tests on each antibiotic against each infection were pooled as shown in tables I, II, III, and IV. From these data, dose-effect lines were drawn and median effective doses were computed. The dose-effect curve parameters and relative potency values are shown in tables V, VI, and VII.

From inspection of either the observed data or the statistical summation, it can be seen that aspartocin is a potent antibiotic when administered parenterally. Against the *Str. pyogenes* C203, *D. pneumoniae* SV1, and *Staph. aureus* Smith infections, aspartocin was more effective intraperitoneally than subcutaneously. When administered at relatively high dosage levels by the oral route (oral tubing or in the diet) against these infections, aspartocin was inactive.

The LD₅₀ values of the sodium salt of aspartocin in mice are shown in table VIII.

SUMMARY

Aspartocin is a new antibiotic produced by *S. griseus* var. *spiralis*. The subcutaneous and intraperitoneal potency of aspartocin has been determined in three standardized infections in mice. In addition, the intravenous potency was measured against *Staph. aureus* Smith (tetracycline sensitive), and the subcutaneous and intravenous activity were determined against *Staph. aureus* Rose (tetracycline resistant).

On an intraperitoneal dosage basis, aspartocin was equal to chlortetracycline against the *Staph. aureus* Smith infection and was 10 to 20 times more potent than

chlortetracycline against both the *Str. pyogenes* C203 and *D. pneumoniae* SV1 infections.

By subcutaneous injection, the new antibiotic was one-fifth as potent as chlor-tetracycline in the *Staph. aureus* Smith infection, about equally potent in the *Str. pyogenes* C203 infection, and about eight times as active in the *Diplococcus* infection. Aspartocin was active both subcutaneously and intravenously against a tetracycline-resistant *Staphylococcus*.

Intravenously, aspartocin was equivalent in activity to tetracycline against the *Staph. aureus* Smith infection.

Aspartocin was inactive orally.

On the basis of acute toxicity studies in mice, aspartocin was approximately two to three times as toxic as tetracycline.

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Fervenulin, a New Crystalline Antibiotic.

I. Discovery and Biological Activities

C. DEBOER, A. DIETZ, J. S. EVANS, AND R. M. MICHAELS

Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

A new crystalline antibiotic, fervenulin, has been isolated from the culture broth of *Streptomyces fervens*, n. sp. This paper is concerned with the discovery and biological properties of fervenulin.

BIOLOGICAL STUDIES

The taxonomic description of *S. fervens*, together with the fermentation, bioassay, papergram, bioautographs, antimicrobial spectra, and in vivo data on fervenulin are reported in this paper. A companion paper on the isolation and characterization of fervenulin will be reported by Eble et al.¹

Taxonomy. This new *Streptomyces* species was isolated from a California soil and was checked for identity with species described in *Bergey's Manual of Determinative Bacteriology*² and by Waksman and Lechevalier.³ Since this isolate appears to differ morphologically and physiologically from any previously described species, the name *S. fervens*, n. sp., is proposed.

This microorganism is characterized by a pink to red vegetative mycelium and a pink aerial growth. Sporophores are scarce and are either monoverticillate or biver-ticillate. Pigment granules are present in the mycelium. This culture, *S. fervens*, has been assigned NRRL no. 2755.

S. fervens was first compared on Ektachrome⁴ and found to be different from the described species in our collection. The characteristics of *S. fervens* on Ektachrome are given in table I.

The carbon utilization pattern of *S. fervens* in the synthetic medium of Pridham and Gottlieb⁵ is given in table II. Additional cultural characteristics of this new species are recorded in table III.

Fermentation. The inoculum was prepared with spores obtained from a maltose-tryptone agar slant and was grown in 500 ml. Erlenmeyer flasks at 28 C. on a reciprocating shaker in 100 ml. of seed medium (glucose, 40 Gm./liter; cotton seed meal, 25 Gm./liter; tap water; pH adjusted to 7.2 prior to sterilization).

After three days' incubation, the vegetative growth was used at the rate of 3 per cent to inoculate a 20 liter seed fermentor containing glucose, 20 Gm./l.; Kay-soy,* 10 Gm./l.; Brewer's yeast, 2.5 Gm./l.; ammonium chloride, 5 Gm./l.; sodium chloride, 3 Gm./l.; calcium carbonate,† 4 Gm./l.; and tap water. The inoculated seed medium was incubated at 28 C. for two days, aerated at the rate of 0.2 cu. ft./min., and agitated with a sweep stirrer.

The contents of the 20 l. seed fermentor were used to inoculate a 100 gallon

* Archer Daniels Midland Co., Cleveland, Ohio.

† Calcium carbonate was added after the medium was adjusted to pH 7.2 and before sterilization.

TABLE I
Appearance of S. fervens on Ektachrome

Agar media	Surface growth	Reverse
Bennett's	Pink aerial	Red
Czapek's sucrose	Trace, pink aerial	Faint pink
Maltose-tryptone	Pink aerial	Red
Peptone-iron	Gray vegetative mycelium	Brown
Tyrosine (0.1 per cent)	Fair, pink aerial	Pink-red
Casein starch	Fair, pink aerial	Red

fermentor containing 250 l. of this medium. Sterile air was supplied at the rate of 2.6 cu. ft./min., and the medium was agitated with a propeller at the rate of 280 r.p.m. Lard oil (900 ml.) was added during the course of the fermentation to control foaming. After three to four days of incubation at 28 C. fervenulin titers of 100 to 200 µg./ml. were obtained.

Assay. A standard agar diffusion assay was devised similar to that of Loo et al⁷ for streptomycin using Penassay seed agar (8 ml./8.5 cm. Petri dish). *Klebsiella pneumoniae* was used as the test organism, and plates were incubated at 37 C. for 16 hours.

A crystalline preparation, dissolved in 0.1 M phosphate buffer at pH 6.0, in concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg./ml., was used as the standard. Figure 1 gives the standard dose-response curve for fervenulin.

Papergram Bioautographs. Papergram bioautographs of the culture filtrate of *S. fervens* disclosed an active component, fervenulin, with an R_f of 0.5 when using 84 per cent 1-butanol and 16 per cent water by volume, containing 0.25 per cent *p*-toluenesulfonic acid as the solvent system and *K. pneumoniae* as the test organism (fig. 2). Papergrams were developed in a descending system. On the basis of papergram bioautographs, fervenulin appeared to be new and different from any of the known antibiotics available for comparison.

In a system consisting of 25 per cent acetic acid, 50 per cent 1-butanol, and 25 per cent water, fervenulin had an R_f of 0.75. In 96 per cent water and 4 per cent 1-butanol it showed an R_f of 0.75.

TABLE II
Assimilation of Carbon Compounds in Synthetic Medium by S. fervens

Positive	Positive (slight growth)	Negative	Negative (slight growth)
D-Glucose	Soluble starch	Sucrose	D-Xylose
D-Mannose	Sodium acetate	Raffinose	L-Arabinose
Maltose	Sodium citrate	Inulin	Rhamnose
Dextrin	Sodium succinate	Dulcitol	D-Fructose
Glycerol		D-Mannitol	D-Galactose
Inositol		D-Sorbitol	Lactose
		Salicin	Cellobiose
		Phenol	Sodium formate
		Cresol	Sodium oxalate
		Sodium salicylate	Sodium tartrate

TABLE III

Cultural Characteristics of S. fervens

Medium	Surface growth	Reverse	Other
Peptone-iron agar	No aerial; gray vegetative	Brown	Hydrogen sulfide darkening
Tyrosine agar (0.1 per cent)	Deep pink aerial	Pink	Fair brown pigment
Litmus milk	Ring at surface; brown on top; cream-pink on bottom; trace, pink aerial		Brown pigment; pH 7.0; no peptonization; no coagulation
Synthetic nitrate broth	Pink surface pellicle; trace pink aerial		Pink-tan growth at base; slight tan pigments; nitrates reduced
Organic nitrate broth	Pink vegetative; ring at surface; no aerial		Flocculent growth at base; tan pigment; nitrates reduced
Plain gelatin (12 per cent)			Brown pigment; one-half liquefied
Calcium malate agar	Trace pink aerial	Pink	No pigment
Glucose asparagine agar	Firm pink aerial	Pink	Pale-yellow pigment
Skim milk agar	No aerial; pink vegetative	Pink-tan	No pigment; no hydrolysis
Casein starch agar	Pink aerial	Pink	Slight tan pigment; starch hydrolyzed
Nutrient starch agar	Slight pink aerial	Red-pink	Tan pigment; starch hydrolyzed
Bennett's agar (18, 24, 28 C.)	Compact pink aerial; plate 5 E-1 6	Red-orange; plate 6 L-9	Slight tan pigment
Bennett's agar (37 C.)	Trace maroon aerial	Maroon	Slight tan pigment
Czapek's sucrose agar (18, 24 C.)	Fair pink-white aerial	Pink-white	No pigment
Czapek's sucrose agar (28, 37 C.)	Fair pink aerial	Pink	No pigment
Maltose-tryptone agar (18 C.)	Compact pink aerial	Red-orange	Slight tan pigment
Maltose-tryptone agar (24, 28 C.)	Peach-pink aerial; plate 3 F-9	Red-brown plate 7 L-10	Slight tan pigment
Maltose-tryptone agar (37 C.)	Trace pink aerial	Maroon	Slight tan pigment

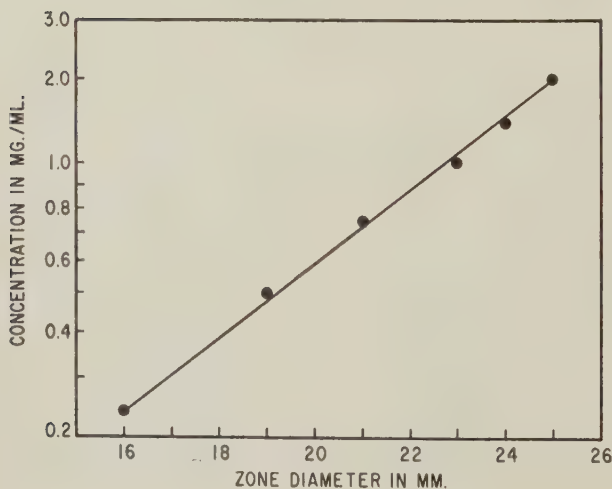
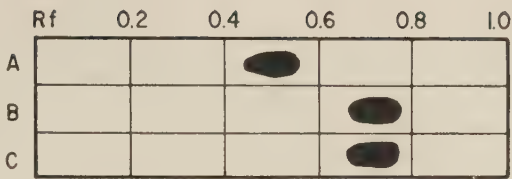


FIG. 1. Standard dose-response curve for fervenulin.

FIG. 2. Papergram bioautograph of the culture filtrate of *Streptomyces fervens*. A, 1-butanol-water, 84:16 (v/v), plus 0.25 per cent (w/v) *p*-toluenesulfonic acid; B, 1-butanol-acetic acid-water, 2:1:1 (v/v); C, 1-butanol-water, 4:96 (v/v).



BIOLOGICAL ACTIVITY IN VITRO

Antiprotozoal Activity. A broth dilution technique was used to determine the minimal inhibitory concentration, using bacto fluid thioglycollate medium plus 5 per cent horse serum (Difco). Fervenuin inhibited *Trichomonas foetus* and *Trichomonas vaginalis* at 20 µg./ml. and *Entamoeba histolytica* at 80 µg./ml.

Tissue Culture. The cytotoxicity of fervenuin to KB human epidermoid carcinoma cells in vitro was determined by the procedure of Smith et al.⁸ The drug concentration that caused 50 per cent inhibition of KB protein synthesis (ID₅₀) was 15 µg./ml., which is marginal when compared with a large series of tissue culture inhibitors.⁹ However, the compound was considered worthy of in vivo anti-tumor evaluation on the basis of its in vitro cytotoxicity.

Antibacterial Activity. A broth dilution spectrum (table IV) revealed that fervenuin was active against *Mycobacterium bovis* (BCG) and gram-negative and gram-positive bacteria. This antibiotic was also active against bacterial plant pathogens, as is evident from the spectrum. The antibacterial plant pathogens were tested in N.I.H. broth (BBL), while the other bacteria were run in brain-heart infusion medium (Difco).

Antifungal Activity. In an agar dilution test, *Histoplasma capsulatum* was in-

TABLE IV
Antibacterial Spectrum of Fervenuin

Test organism	Minimal inhibitory concentration, µg./ml.
<i>Pasteurella multocida</i>	3.1
<i>Salmonella pullorum</i>	25
<i>Aerobacter aerogenes</i>	25
<i>Bacillus subtilis</i>	50
<i>Salmonella typhosa</i>	50
<i>Salmonella paratyphi</i> B	50
<i>Escherichia coli</i>	50
<i>Klebsiella pneumoniae</i>	25
<i>Mycobacterium bovis</i> (BCG)	50
<i>Diplococcus pneumoniae</i>	100
<i>Streptococcus hemolyticus</i>	10
<i>Staphylococcus albus</i>	100
<i>Proteus vulgaris</i>	100
<i>Staphylococcus aureus</i>	1
<i>Streptococcus viridans</i>	100
<i>Agrobacterium tumefaciens</i>	200
<i>Corynebacterium fascians</i>	100
<i>Corynebacterium michiganensis</i>	100
<i>Erwinia amylovora</i>	200
<i>Xanthomonas campestris</i>	200
<i>Xanthomonas pelargonii</i>	200
<i>Xanthomonas vesicatoria</i>	200
<i>Xanthomonas malvacearum</i>	>200

TABLE V

*Antifungal Spectrum of Fervenuin**

Test organism	Minimal inhibitory concentration, μg./ml.
<i>Nocardia asteroides</i>	1000
<i>Blastomyces dermatitidis</i>	100
<i>Coccidioides immitis</i>	1000 (100 partial inhibition)
<i>Geotrichum</i> sp.	1000
<i>Hormodendrum compactum</i>	1000
<i>Cryptococcus neoformans</i>	100
<i>Histoplasma capsulatum</i>	10
<i>Sporotrichum schenckii</i>	1000
<i>Monosporium apiospermum</i>	1000
<i>Microsporium audouini</i>	100
<i>Candida albicans</i> Abbott	>1000
<i>Candida albicans</i> McKinney	>1000
<i>Candida albicans</i> Upjohn	>1000
<i>Microsporium canis</i>	100
<i>Trichophyton interdigitale</i>	100

* Fungal spectrum agar in Gm./l of distilled water: dextrose, 10; bacto peptone, 5; yeast extract, 1; bacto agar, 20. Adjust pH to 6.8.

hibited at 10 μg./ml., while *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Microsporium audouini*, *Microsporium canis*, and *Trichophyton interdigitale* were inhibited at 100 μg./ml. The antifungal spectrum is given in table V.

BIOLOGICAL ACTIVITY IN VIVO

Fervenuin was tested for antitrichomonal activity in mice and hamsters. Experimental infections with *T. vaginalis* and *T. foetus* were established in mice by subcutaneous injections. In hamsters, experimental infections of *T. foetus* were established intravaginally.

In therapeutic trials, fervenuin was administered to mice orally, intraperitoneally, and subcutaneously at the site of infection. The results, expressed as the curative

TABLE VI

Antitrichomonal Activity of Fervenuin in Mice

Species	Drug route	No. of daily treatments	CD ₅₀ , mg./Kg./day	LD ₅₀ , mg./Kg.
<i>T. vaginalis</i>	Subcutaneous	6	3.4	65
	Oral	6	4.4	
	Intraperitoneal	1	7.5	
	Intraperitoneal	3	5.4	
	Intraperitoneal	5	4.1	
	Intraperitoneal	6	3.9	
<i>T. foetus</i>	Subcutaneous	6	2.5	
	Oral	6	3.5	
	Intraperitoneal	6	3.0	

TABLE VII

Antitrichomonal Activity of Fervenuin in Hamsters Treated Daily for 14 Days

Drug route	Drug dose, mg./Kg./day	Per cent cured	LD ₅₀ , mg./Kg.
Intraperitoneal	4	75	11.2
Intraperitoneal	2	0	
Subcutaneous	4	0	
Subcutaneous and intraperitoneal*	4 and 4	84	

* Combined therapy by both routes.

dose protecting 50 per cent of the animals (CD_{50}), together with toxicity data in the test animal, are compiled in table VI.

In infected hamsters, fervenuin was administered both intraperitoneally and intravaginally. In addition, therapy was attempted by administering fervenuin simultaneously by both routes. As shown in table VII, a cure rate of 75 per cent was attainable by intraperitoneal therapy. This success rate could not be substantially enhanced by simultaneous administration of an equal dose of drug at the site of infection. The significant failure rate could not be attributed to development of resistant mutants under therapy, since *T. foetus*, isolated before and after drug administration, was essentially unchanged in its sensitivity to fervenuin.

Antitumor Activity. Fervenuin has been tested in 4 rat and 2 mouse tumor systems. The antibiotic was inactive against sarcoma 180, Ehrlich carcinoma ascites, Walker adenocarcinoma, Murphy-Sturm lymphosarcoma, Jensen sarcoma, and Guerin adenocarcinoma, when tested at 10 and 20 mg./Kg./day, using previously described methods.^{10 12} It was impossible to administer routinely the higher dose level because of emaciation of the animals due to anorexia and diarrhea. The ineffectiveness of the antibiotic could have been predicted from the acute LD₅₀ and the cytotoxicity of the drug against cells in tissue culture, if one assumes a correlation between tumor inhibition and tissue culture cytotoxicity.

Antimicrobial Activity. There was no demonstrable activity against *Streptococcus hemolyticus* and *K. pneumoniae* in mice at 12.5 mg./Kg. when fervenuin was administered either orally or subcutaneously. Fervenuin was inactive against *Histoplasma capsulatum* and *Cryptococcus neoformans* in mice up to 10 minimal bactericidal doses. It was also inactive in mice at 6.25 mg./Kg. against influenza A (PR-8) virus.

SUMMARY

A new antibiotic, fervenuin, was discovered in the culture filtrate of *Streptomyces fervens*, n. sp. The morphology and physiology of this new species are described. An agar plate assay and papergram bioautographs using *Klebsiella pneumoniae* as the test organism are given. The antibiotic demonstrated broad-spectrum antibacterial, antifungal, antiparasitic, and antitumor cell activity in vitro. Fervenuin has a CD_{50} of 3.9 mg./Kg., intraperitoneally, against *Trichomonas* in hamsters and mice.

ACKNOWLEDGMENTS

Grateful acknowledgment is given to Dr. S. P. Owen and Mr. J. Visser for tank fermentations; Dr. W. T. Sokolski and Miss N. J. Eilers for paper chromatography studies; Drs. C. G. Smith, J. E. Grady, and Mr. C. M. Large for tissue culture data; and Dr. L. J. Sorenson and Mr. C. Lewis for in vivo data.

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Fervenulin, a New Crystalline Antibiotic.

II. Isolation and Characterization

T. E. EBLE, E. C. OLSON, C. M. LARGE, AND J. W. SHELL

Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

The isolation and properties of a new antibiotic, fervenulin, are described in this report. The biological activity of the antibiotic, which is derived from culture filtrates of a new actinomycete, *Streptomyces fervens*, is reported in a companion paper.¹

ISOLATION

Crystalline fervenulin can be obtained by the following procedure: The whole broth is adjusted to approximately pH 8 and filtered. The clear filtrate is adjusted to approximately pH 6 and extracted with one fourth volume of methylene chloride. The extract is concentrated to an oil, which is leached with upper phase of a solvent system consisting of acetone, *n*-hexane, and water in the volume ratio of 5:3:1. The leached extract is concentrated to afford crystalline fervenulin.

Pure fervenulin can be obtained by repeated fractional crystallization from the solvent pair ethyl acetate:acetone (volume ratio 3:1), or by countercurrent distribution in the system benzene:methanol:water (1:1:0.2).

CHARACTERIZATION

Fervenulin exists as brilliant yellow orthorhombic crystals. It melts with decomposition at 178 to 179 C. and sublimes readily at 70 C. and 10 μ pressure.

The empirical formula for fervenulin was established as $C_7H_7N_5O_2$ by the analytical data (calculated: C, 43.52; H, 3.65; N, 36.26; O, 16.56; found: C, 43.83; H, 3.73; N, 35.99; O, 17.27). The molecular weights, as shown in table I, indicate that this is also the molecular formula.

The infrared spectrum for fervenulin is shown in figure 1. The spectrum in mineral oil suspension shows characteristic absorption at the following frequencies: 3040, 1718, 1675, 1575, 1535, 1495, 1435, 1415, 1397, 1296, 1255, 1219, 1155, 1085, 1044, 995, 962, 930, 884, 815, 740, 730, and 709 cm^{-1} . This spectrum is of particular interest in that it shows a complete absence of any absorption in the OH/NH region. The single carbonyl absorption at 1718 cm^{-1} coupled with the doublet of nearly equal intensity at 1296 and 1219 cm^{-1} in the C-O region suggests the presence of a six-membered lactone. Other data indicate that fervenulin is an enol lactone.

The ultraviolet spectrum, when run in ethanol or pH 7.85 phosphate buffer (fig. 2), exhibits a maximum at 239 $m\mu$ ($a = 87$), a small maximum between 270 and 280 $m\mu$ ($a = 8.5$ to 9.0), and a maximum at 340 $m\mu$ ($a = 23$). At pH 10 the maxima at 239 and 340 $m\mu$ are destroyed, while the maximum at 270 to 280 $m\mu$ is unchanged.

TABLE I

Molecular Weight of Fervenuin
(Theory for $C_7H_7N_3O_2$: 193.17)

Method	Found
Radiological study	189
Vapor pressure	190
Saponification	186

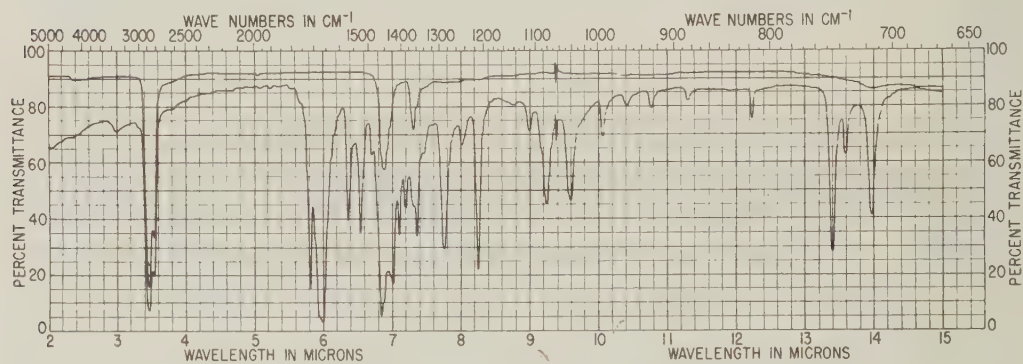


FIG. 1. Infrared spectrum of fervenuin in mineral oil suspension.

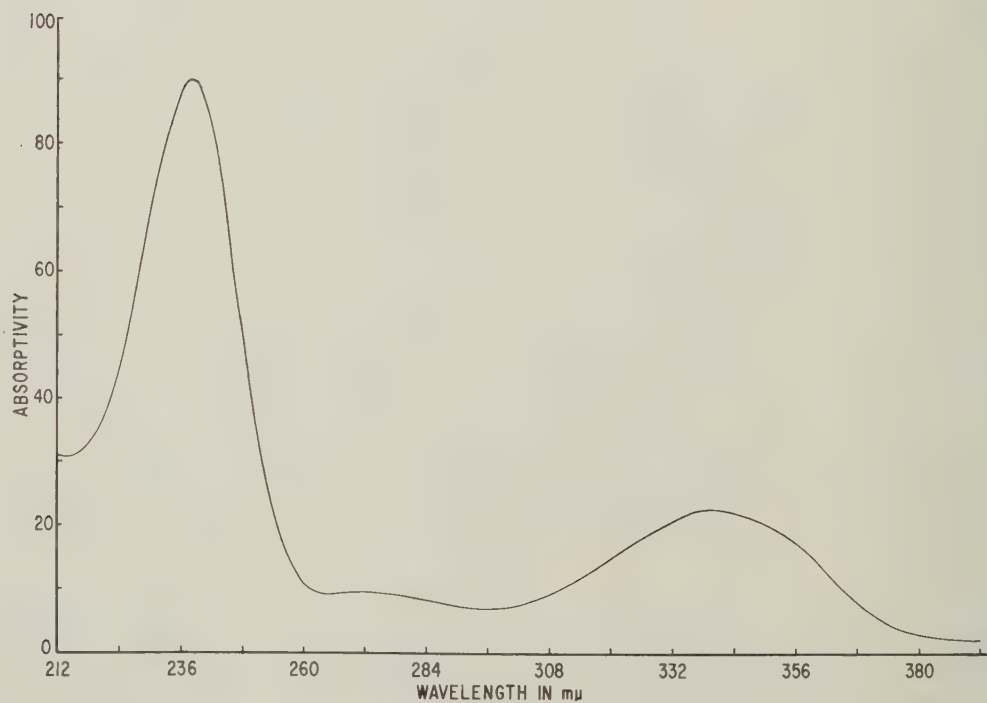


FIG. 2. Ultraviolet spectrum of fervenuin.

TABLE II
Crystal Properties of Fervenulin

Crystal system:	Orthorhombic
Crystal class:	Rhombic dipyramidal
Crystal habit:	Lamellar
Axial ratio:	a:b:c, 0.883:1.0:0.316
Unit cell dimensions:	a = 15.86 Å b = 17.96 Å c = 5.667 Å
Crystal density:	1.55
Formula weights per cell:	8
Optic sign:	Negative
Dispersion:	Extreme V > R
Optic axial angle:	2V = 23° 30' (calc.)
Optic orientation:	b = X, c = Y, a = Z
Refractive indexes (for sodium light)	a = 1.584 β = 1.706 γ = 1.712

Fervenulin has crystallographic properties as shown in table II. It is reducible at the dropping mercury electrode and gives the following waves (versus S.C.E.): $E_{1/2} = +0.06$ and -0.85 V. in 2 *N* sulfuric acid, $E_{1/2} = -0.22$ and -1.03 V. in pH 3 buffer, and $E_{1/2} = -0.057$, -1.32 , and -1.62 V. in pH 8 buffer. The changes in wave height with pH are such as to eliminate the consideration of the presence of a nitro group in the compound.

Fervenulin is a neutral compound soluble in practically all of the common organic solvents and insoluble in the hydrocarbons. It is soluble in cold water to about 2 mg./ml. and in hot water to about 40 mg./ml.

Fervenulin is labile to alkali but is extremely stable to acid, having been recovered intact after treatment with 6 *N* hydrochloric acid on a steam bath for 40 hours.

Fervenulin gives a positive copper sulfide test for hydrocyanic acid but negative ninhydrin, ferric chloride, Tollen's, Benedict's iodoform, biuret, Hinsberg, and Sakaguchi tests.

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Streptozotocin, a New Antibacterial Antibiotic

J. J. VAVRA, C. DEBOER, A. DIETZ, L. J. HANKA, AND W. T. SOKOLSKI

Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

Streptozotocin is a new antibiotic produced by *Streptomyces achromogenes* var. 128. The producing organism was isolated from a soil sample taken at Blue Rapids, Kansas, and has been deposited with the Northern Utilization Research Branch, U.S. Department of Agriculture, as culture NRRL 2697. Streptozotocin is active against a variety of gram-positive and gram-negative organisms both in vitro and in vivo. This report deals with the taxonomy of the producing organism, fermentation conditions necessary for streptozotocin production, papergram identification, and in vitro activity of this antibiotic. Other papers will describe extraction and characterization studies,¹ in vivo mouse activity,² resistance studies,³ and a blood assay method.⁴

MATERIALS AND METHODS

Taxonomy studies were based on Ektachrome comparisons,⁵ carbon utilization studies,⁶ growth characteristic studies, and microscopic appearance.

Stock cultures of *S. achromogenes* var. 128 were maintained as spore preparations on sterile soil. Vegetative seed for fermentation studies was prepared by incubating spores from a soil stock for 48 hours in a seed medium containing glucose monohydrate, 25 Gm./liter, and cottonseed flour, 25 Gm./liter. Presterilization pH was 7.2 and the production flasks were inoculated at the rate of 0.5 to 2.0 ml. vegetative seed per 100 ml. growth medium in a 500 ml. Erlenmeyer flask. All fermentation flasks were incubated on a Gump rotary shaker operating at 250 r.p.m. with a two inch stroke. Fermentation temperature was maintained at 28 C. unless specified otherwise. Media were sterilized at 121 C. for 20 minutes prior to inoculation. Assays were run on the supernatant from centrifuged beers using a disc plate, agar diffusion method. The test organism was *Proteus vulgaris* and the test medium was nutrient agar (Difco). A crystalline streptozotocin sample was used as the standard.

Due to the instability of streptozotocin at neutral or alkaline conditions,⁴ special procedures had to be devised to obtain data on the in vitro spectrum of this antibiotic. In this test, tubes containing 10 ml. nutrient broth (Difco) were inoculated with 1 drop of a 20 hour culture and were incubated for one to two hours at 37 C. The optical density was measured (650 m μ) and antibiotic was added to give final concentrations in the tubes of 0.1 to 20 μ g./ml. The tubes were incubated for an additional six hours and the amount of growth was measured turbidimetrically. Minimal optical density was that value observed at the time of addition of the antibiotic. Maximal optical density was the value for the control tube at the end of the experiment. Inhibition was calculated as the concentration of drug that allowed growth of the organism to reach 50 per cent of the maximal optical density.

Streptozotocin was differentiated from other known antibiotics by descending paper chromatography. Approximately 5 μ g. was spotted on Whatman no. 1 filter

TABLE I

Macroscopic Comparison of Cultures on Ektachrome Transparencies

Medium	<i>S. achromogenes</i>			
	Variant 128		Waksman no. 3730	
	Surface	Reverse	Surface	Reverse
Bennett's	Gray	Brown	Gray	Brown
Czapek's sucrose	Gray	Tan	Gray	Tan
Maltose tryptone	Gray-white	Brown	Gray-white	Brown
Peptone iron	No aerial growth	Brown	No aerial growth	Brown
0.1% tyrosine	Gray-white	Brown	Gray	Brown
Casein starch	Gray	Brown	Gray	Brown

TABLE II

Utilization of Carbon Compounds in Synthetic Medium by Strains of S. achromogenes

Carbon source	<i>S. achromogenes</i>	
	Variant 128	Waksman no. 3730
Control	—*	(—)
D-Xylose	+	+
L-Arabinose	+	+
Rhamnose	+	+
D-Fructose	+	+
D-Galactose	+	+
D-Glucose	+	+
D-Mannose	+	+
Maltose	+	+
Sucrose	(—)	(—)
Lactose	+	+
Cellobiose	(+)	+
Raffinose	(—)	(—)
Dextrin	(+)	+
Inulin	(—)	(—)
Soluble starch	(+)	+
Glycerol	+	+
Dulcitol	(—)	(—)
D-Mannitol	+	+
D-Sorbitol	(+)	(+)
D-Inositol	(—)	(+)
Salicin	(+)	+
Phenol	—	—
Cresol	—	—
Sodium formate	—	(—)
Sodium oxalate	—	—
Sodium tartrate	—	(—)
Sodium salicylate	—	—
Sodium acetate	+	+
Sodium citrate	(+)	+
Sodium succinate	+	+
All cases	Pink to gray aerial growth	

*+ = Positive assimilation. (+) = Positive assimilation, slight growth. (—) = Slight growth, no assimilation. — = No growth.

TABLE III
Growth Characteristics of Strains of S. achromogenes

Medium	<i>S. achromogenes</i>	
	Variant 128	Waksman no. 3730
Plain gelatin	Gray aerial growth Brown pigment Trace liquefaction	Gray aerial growth Brown pigment No liquefaction
Nutrient gelatin	No aerial growth Brown pigment Trace liquefaction	White aerial growth Brown pigment No liquefaction
Nutrient nitrogen broth	Gray-white aerial growth on surface pellicle Yellow-tan pigment Reduction negative*	Gray-white aerial growth on surface pellicle Yellow-tan pigment Reduction negative
Synthetic nitrogen broth	Vegetative growth—pellicle at surface and flocculent throughout Trace gray-white aerial growth Trace yellow pigment Reduction negative*	Vegetative growth—pellicle at surface and flocculent throughout Trace gray-white aerial growth Strong yellow pigment Reduction negative
Tryptone broth	Trace gray-white aerial growth on surface colonies Brown-tan pigment	Trace gray-white aerial growth on surface colonies Brown-tan pigment
Litmus milk	No change	No change
Tyrosine agar	Gray aerial growth Gray reverse Brown pigment	Gray aerial growth Gray reverse Pink-tan pigment
Peptone iron agar	Colorless vegetative growth Hydrogen sulfide darkening	Colorless vegetative growth Hydrogen sulfide darkening
Calcium malate agar	Gray aerial growth Gray reverse	Gray-white aerial growth Gray-white reverse
Glucose asparagine agar	Gray-white aerial growth Cream reverse Yellow pigment	Gray-white aerial growth Cream reverse Yellow pigment
Maltose-tryptone agar	Gray-blue aerial growth Brown reverse Brown pigment	Gray-blue aerial growth Brown reverse Brown pigment
Bennett's agar, 18 C.	Fair gray-pink aerial growth tinged with green Tan reverse Tan pigment	Fair gray-pink-cream aerial growth Tan reverse Tan pigment
Bennett's agar, 24 C.	Fair gray-white aerial growth Tan reverse Tan pigment	Fair gray-white aerial growth Tan reverse Tan pigment
Bennett's agar, 28 C.	Gray-pink aerial growth Tan reverse Tan pigment	Gray-pink aerial growth Tan reverse Tan pigment
Bennett's agar, 37 C.	Good gray-blue-tan aerial growth Brown-tan reverse Brown-tan pigment	Good gray-blue-tan aerial growth Brown-tan reverse Brown-tan pigment
Bennett's agar, 55 C.	No growth	No growth
Czapek's sucrose agar, 18 C.	Trace gray aerial growth Gray reverse	Trace gray aerial growth Gray reverse
Czapek's sucrose agar, 24 C.	Fair gray aerial growth Gray reverse	Fair gray aerial growth Gray reverse
Czapek's sucrose agar, 28 C.	Fair gray aerial growth Gray reverse	Fair gray aerial growth Gray reverse
Czapek's sucrose agar, 37 C.	Good gray aerial growth Gray reverse	Good gray aerial growth Gray reverse

Table III Continued on Page 233

TABLE III (Continued)
Growth Characteristics of Strains of *S. achromogenes*

Medium	<i>S. achromogenes</i>	
	Variant 128	Waksman no. 3730
Czapek's sucrose agar, 55 C.	No growth	No growth
Casein starch agar	Lavender-gray aerial growth Tan reverse Hydrolysis	Lavender-gray aerial growth Tan reverse Hydrolysis
Nutrient starch agar	Pink-white aerial growth Cream-tan reverse Hydrolysis	Pink-white aerial growth Cream-tan reverse Hydrolysis

* Checked for nitrate reduction at 24 and 48 hours.

paper, and development was made with no equilibration in the solvent vapors. The activity was located by placing developed strips on trays of agar seeded with *P. vulgaris*, *Staphylococcus aureus*, or *Escherichia coli*.

RESULTS AND DISCUSSION

Taxonomy. From taxonomic studies, the culture producing streptozotocin appears to be a variant of *S. achromogenes* Waksman 3730. This organism, however, does not produce streptozotocin and does not produce the brown pigment characteristically produced by *S. achromogenes* var. 128 in most media. An Ektachrome comparison of the two cultures is given in table I. Table II presents the utilization of carbon compounds by *S. achromogenes* var. 128 in the synthetic medium of Pridham and Gottlieb. Growth characteristics of the producing culture are given in table III. Incubation was carried out at 28 C. unless otherwise noted. *S. achromogenes* var. 128 characteristically produces sporophores that are mostly straight, with some having open loops.

TABLE IV
In Vitro Spectrum of Streptozotocin

Organism	Strain	IC ₅₀ *, µg./ml.
<i>Salmonella pullorum</i>	MSDH 75	0.15
<i>Proteus vulgaris</i>	ATCC 6380	0.20
<i>Escherichia coli</i>	ATCC 26	0.50
<i>Salmonella typhi</i>	MSDH TG 3	0.90
<i>Streptococcus faecalis</i>	ATCC 6057	2.0
<i>Klebsiella pneumoniae</i>	University of California A-D	3.0
<i>Staphylococcus aureus</i>	OSU 284	0.75
<i>Salmonella schottmülleri</i>	ATCC 9149	0.35
<i>Pasteurella multocida</i>	Lederle P-449	<0.25
<i>Aerobacter aerogenes</i>	ATCC 8308	10.0
<i>Pseudomonas aeruginosa</i>	ATCC 9027	>50.0

* Concentration of streptozotocin that allows growth of the organism to reach 50 per cent of the possible maximum.

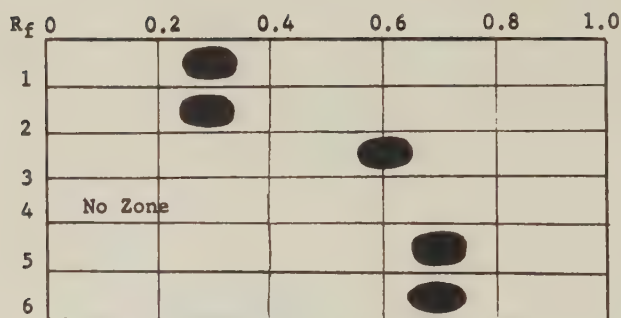


FIG. 1. Papergram pattern of streptozotocin. 1, *n*-butanol:water 84:16 (v/v); 2, *n*-butanol:water 84:16 plus 0.25 per cent (w/v) *p*-toluenesulfonic acid; 3, *n*-butanol:acetic acid:water 2:1:1 (v/v); 4, *n*-butanol:water 84:16; 2 ml. piperidine added to 98 ml. of butanol-water mixture; 5, water-*n*-butanol, 96:4 (v/v); 6, water-*n*-butanol, 96:4 plus 0.25 per cent *p*-toluenesulfonic acid.

Fermentation Studies. Medium studies showed that good yields of streptozotocin were obtained when *S. achromogenes* var. 128 was grown for six to seven days in a basal medium of the composition: Cerelose (glucose monohydrate) 3; starch, 15; refined corn meal, 40; peptone (Difco), 3 Gm./liter. Optimal antibiotic production was obtained with seed prepared from a soil stock and a temperature cycle of four days at 32 C. followed by two days at 28 C. Under these conditions, peak yields of 350 to 450 µg./ml. of streptozotocin were obtained. Substitutions of the following nitrogen sources for refined corn meal gave decreased titers: ground corn, soybean meal, soy flour, corn gluten meal, fish meal, and corn steep liquor. Substitution of Wilson's peptone 159 and NZ-amine B (Sheffield) for Difco peptone gave production of streptozotocin comparable to that of the control. On the other hand, substitution of Difco peptone with Amber BYF (yeast autolysate), soy peptone (Sheffield), peptonized milk nutrient (Sheffield), ammonium sulfate, or milorganite gave decreased yields.

Optimal production of streptozotocin was obtained when the pH of the fermentation remained in the range of 4.5 to 6.0. A pH rise results in destruction of the antibiotic.⁴ Many of the low titers obtained when the ingredients just listed were used probably due to a pH rise.

Addition of ammonium sulfate at 2 to 5 Gm./liter to fermentation media often helped to stabilize the pH at a value within the range of optimal production of streptozotocin. Utilization of the NH₄⁺ caused liberation of SO₄⁼, which helped to lower the pH of the fermentation.

In Vitro Activity. The in vitro activity of streptozotocin against a variety of organisms is given in table IV. As can be seen from table IV, streptozotocin inhibits both gram-positive and gram-negative organisms in vitro.

Cross Resistance. Streptozotocin demonstrated no cross resistance with the following antibiotics when tested against induced and clinically resistant strains of *Staph. aureus*: novobiocin, carbomycin, celesticetin, chloramphenicol, erythromycin, kanamycin, neomycin, penicillin, polymyxin, and tetracycline.

Paper Chromatography. The papergram pattern of streptozotocin obtained after development with six solvent systems is given in figure 1.

SUMMARY

1. Streptozotocin is a new antibiotic produced by a streptomycete isolated from the soil. The organism is a variant of *S. achromogenes* and is designated *S. achromogenes* var. 128.

2. Medium studies have shown that streptozotocin can be produced in yields in excess of 400 $\mu\text{g./ml.}$ in a medium containing refined corn meal and a soluble peptone as nitrogen sources and starch and glucose as carbon sources. Use of seed inoculated from a soil stock and a temperature shift during the fermentation resulted in optimal production.

3. Streptozotocin is active against both gram-positive and gram-negative organisms and is not cross resistant with 10 commercially available antibiotics. Due to the instability of streptozotocin at neutral and alkaline pH , special techniques had to be used to define its *in vitro* spectrum.

4. Paper chromatographic identification of streptozotocin is given.

ACKNOWLEDGMENTS

The authors are indebted to Mr. Walter Murphy, Mr. Carl O. Schwartz, Miss N. J. Eilers, Mr. E. D. Rogers, Mr. M. R. Burch, and Mr. J. A. Santek for technical assistance and to Drs. S. P. Owen, B. W. Churchill, and H. J. Koepsell and to Mr. J. Visser for helpful suggestions and encouragement given throughout the course of this work.

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Isolation and Characterization of Streptozotocin

R. R. HERR, T. E. EBLE, M. E. BERGY, AND H. K. JAHNKE

Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

This paper concerns the isolation and purification of a new broad-spectrum antibiotic, streptozotocin, from a *Streptomyces achromogenes* fermentation broth. Some chemical and physical properties of the crystalline material are described. Biological properties of streptozotocin are described in companion papers.¹⁻⁴

ISOLATION

Crystalline streptozotocin has been isolated by the following procedure: The whole broth was acidified to pH 4.0 and filtered. The clear broth was concentrated under reduced pressure to 0.08 volume and added to 5 volumes of acetone. After removal of the precipitate by filtration, the acetone filtrate was concentrated to an aqueous solution and freeze-dried. This crude product was purified by partition chromatography on a Dicalite column using the solvent system of 1-butanol, cyclohexane, and pH 4.0 buffer in the volume ratio 20:4:4. The product from the peak fractions was again chromatographed on Dicalite using the solvent system of methyl ethyl ketone, cyclohexane, and pH 4.0 buffer in the volume ratio 9:1:1.43. The bioactive material from this column was countercurrently distributed for 775 transfers between methyl ethyl ketone and water. Fractions were pooled on the basis of microbiological activity, and the water was removed by azeotropic distillation. Streptozotocin crystallized from the anhydrous methyl ethyl ketone solution. Recrystallization was effected from 95 per cent ethanol.

The isolation of streptozotocin was accomplished using *Proteus vulgaris* as the assay organism.

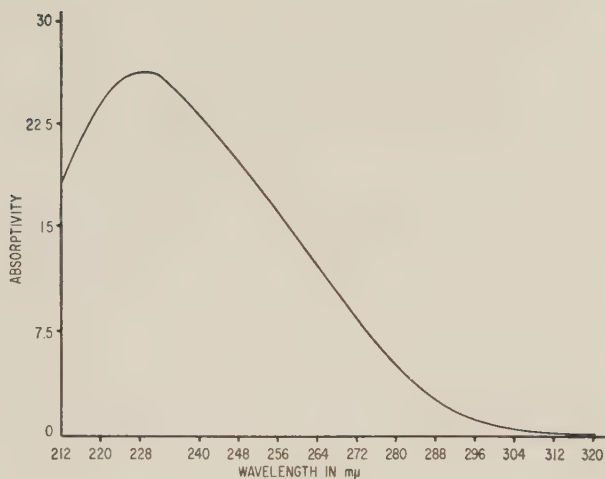
CHARACTERIZATION

Crystalline streptozotocin, isolated as just described, is a mixture either of several crystal forms, not readily interconvertible, or of several closely related compounds, which have resisted attempts at separation. Two obvious crystal types are visible on recrystallization from ethanol, and may be separated by fractional crystallization from this solvent. Both forms, however, are shown to be mixtures by roentgenographic crystallography. Only minor differences have been observed in the properties of the two forms. Both forms show approximately equal activity against the assay organism.

Streptozotocin is very soluble in water, soluble in the lower alcohols, and relatively insoluble in less polar organic solvents.

Streptozotocin has no definite melting point. It decomposes with evolution of gas at about 115 C. and becomes a clear liquid by 125 C. Results of elemental analyses and molecular weight determinations suggest the empirical formula $C_{14}H_{27}N_5O_{12}$. Ultraviolet (fig. 1), and visible absorption studies in ethanol show a strong maximum at 228 m μ ($a = 24$) and very weak maxima at 380 m μ ($a = 0.37$),

FIG. 1. The ultraviolet spectrum of streptozotocin in 95 per cent ethanol.



394 $m\mu$ ($a = 0.48$), and 412 $m\mu$ ($a = 0.40$). The infrared spectrum of a crystalline sample in mineral oil suspension shows multiple OH/NH, at least one carbonyl, and multiple absorption bands in the 1500 cm^{-1} region (fig. 2). The antibiotic is dextrorotatory, and rotatory dispersion measurements show no maximum. Titrations show streptozotocin to have neither acidic nor basic groups.

Samples of dry crystalline streptozotocin stored at 70 C. degraded rapidly and in a random manner. At room temperature, fully active samples were present after 30 days, while at 4 C. the material was stable for periods up to six months. Streptozotocin exhibits maximum stability at pH 4, with stability decreasing rapidly at either higher or lower pH.

In 10 per cent aqueous sodium hydroxide, streptozotocin decomposes immediately with effervescence. When this reaction was carried out at room temperature, the evolved gas appeared to be nitrogen, suggesting the presence of a N-nitroso group. This was supported by a positive Liebermann nitroso test.⁵ The antibiotic also gave negative Benedict, ninhydrin, and biuret tests.

Alkaline degradation of streptozotocin at 0 C. allowed identification of the evolved gas as diazomethane. Acidification of the remaining solution caused evolution of carbon dioxide. The residue is readily oxidized by periodate, giving both

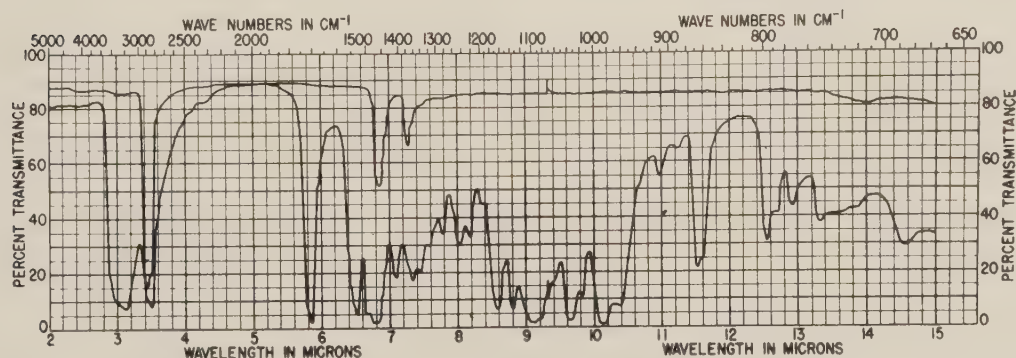


FIG. 2. The infrared spectrum of streptozotocin in mineral oil suspension.

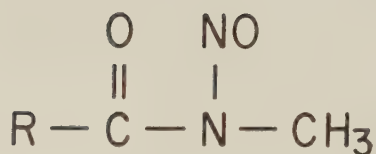


FIG. 3. Suggested N-nitrosomethylamide function in streptozotocin.

formaldehyde and formic acid as products. These observations suggest the presence of a N-nitrosoamide grouping, as shown in figure 3. A colorimetric assay has been developed based on a reaction of the N-nitroso group.⁶

EXPERIMENTAL STUDIES

Isolation from Fermentation Broth. Two hundred fifty liters of a culture broth of streptozotocin was adjusted to pH 4.0, mixed with 10 Kg. of Dicalite 4200, and filtered. The filtered broth was concentrated under reduced pressure to a volume of 20 liters. The concentrate was added to a stirred suspension of 10 Kg. of Dicalite 4200 in 100 liters of acetone, and the resulting mixture was filtered. The filtrate was concentrated to an aqueous solution and freeze-dried to give 575 Gm. of material containing 23 Gm. of streptozotocin (potency 40 µg./mg., yield 44 per cent).

Partition Chromatography—Butanol Column. The solvent system used consisted of 1-butanol, cyclohexane, and MacIlvaine's pH 4.0 buffer in the volume ratio 20:4:4. Fifty Kg. of Dicalite 4200 was mixed with enough upper phase to allow good agitation and 20 liters of lower phase was added slowly. The mixture was poured into a 13 inch diameter stainless steel column and packed to a height of 6 ft. with 5 lb./sq. in. air pressure.

The starting material, 575 Gm. of crude streptozotocin prepared as described previously, was dissolved in 1250 ml. of lower phase and added to a stirred mixture of 2.5 Kg. of Dicalite in 12 liters of upper phase. The resulting mixture was poured evenly on top of a 2 inch bed of sea sand that had been placed on top of the packed Dicalite bed. The column was eluted with upper phase at the rate of 340 ml./minute. A total of 600 liters of effluent was collected in 4 liter fractions. The active fractions were pooled, concentrated to aqueous solutions, extracted with 0.1 volume of 1-butanol, and the aqueous solutions were freeze-dried.

The most active fraction weighed 27 Gm., assayed 185 µg./mg., and represented 22 per cent of the starting activity. Other less potent fractions accounted for an additional 34 per cent.

Partition Chromatography—Methyl Ethyl Ketone Column. The solvent system used consisted of methyl ethyl ketone, cyclohexane, and MacIlvaine's pH 4.0 buffer

TABLE I
Pooling of Active Fractions

Pool	Fraction no.	Wt., Gm.	Potency, µg./mg.	Per cent starting activity
I	435-499	0.68	200	2.7
II	500-610	3.30	850	55.5
III	611-645	0.71	650	9.1
IV	646-710	1.08	100	2.0

in the volume ratio 9:1:1.43. The procedure used to prepare the column was the same as described for the butanol column, using 600 Gm. of Dicalite holding 240 ml. of lower phase in a 2 inch glass column.

The starting material was prepared by dissolving 24.2 Gm. of the product from the butanol column in 20 ml. of lower phase. This solution was mixed with 40 Gm. of Dicalite and 50 ml. of upper phase, and the mixture was poured gently onto the top of the prepared column bed. The column was developed with upper phase at the rate of 6 ml./minute. Twenty ml. fractions were collected. Fractions were analyzed by determination of solids and plating dipped filter paper discs on agar seeded with *P. vulgaris*. Active fractions were pooled as shown in table I and the solvents removed under reduced pressure.

Countercurrent Distribution. Pools II and III (4.0 Gm.) from the partition column described previously were combined with 2.5 Gm. of other chromatographed materials to give 6.5 Gm. of dried product with a potency of 650 µg./mg. This material was countercurrently distributed for 775 transfers between methyl ethyl ketone and water. Fractions 120 to 170 (3.55 Gm. solids) were pooled and concentrated under reduced pressure to an aqueous solution of 100 ml. volume. This solution was then azeotropically distilled in vacuo five times with 100 ml. portions of methyl ethyl ketone. Streptozotocin crystallized from the anhydrous methyl ethyl ketone solution. After refrigeration, the crystals were collected by filtration and dried to give 1.10 Gm. of the crystalline antibiotic. A portion of this product was recrystallized twice from 95 per cent ethanol and submitted for analysis.

Analysis. Calculated for $C_{14}H_{27}N_5O_{12}$: C, 36.76; H, 5.95; N, 15.31; O, 41.98; molecular weight 457; found: C, 36.30; H, 5.74; N, 15.11; O, 39.90; molecular weight (isothermal distillation in acetone) 481, 468.

Alkaline Degradation of Streptozotocin. Two Gm. of streptozotocin was added in portions while stirring to 20 ml. of cold (0 C.) 40 per cent sodium hydroxide solution, which was covered with 70 ml. of ether. After addition was complete, the mixture was stirred for 30 minutes. The green-yellow ethereal phase was separated and dried over potassium hydroxide pellets.

The ether solution was chilled in an ice bath and a slurry of 1 Gm. of *p*-nitrobenzoic acid in ether was added. After the solution had turned colorless and no more gas was evolved, the ether solution was extracted five times with sodium bicarbonate solution and washed three times with water. The dried solution was evaporated to give 508 mg. of crystalline material melting at 96.5 to 97.0 C. A sample of authentic methyl *p*-nitrobenzoate prepared from diazomethane and *p*-nitrobenzoic acid melted at 97.5 to 98.0 C. A mixture of the two samples melted at 97.5 to 98.0 C. Infrared spectra showed the two materials to be identical.

In another experiment, the alkaline solution after the decomposition (under nitrogen) was acidified with 50 per cent sulfuric acid in a stream of nitrogen. The gas was bubbled through barium hydroxide solution. Barium carbonate precipitated, indicating the evolved gas to be carbon dioxide.

SUMMARY

The isolation and characterization of a new broad-spectrum antibiotic, streptozotocin, have been described. Analytical results on a crystalline sample suggest the

empirical formula $C_{14}H_{27}N_5O_{12}$. Chemical studies indicate the antibiotic to possess a N-nitrosomethylamide group.

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Assay Methods and Antibacterial Studies on Streptozotocin

W. T. SOKOLSKI, J. J. VAVRA, AND L. J. HANKA

Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Streptozotocin is a new broad-spectrum antibiotic produced by a variant of *Streptomyces achromogenes* var. 128. Some of its chemical and biological properties have been described.¹⁻⁴

Because streptozotocin is a relatively unstable antibiotic, problems were encountered in devising a biological assay. This report deals with stability and bactericidal studies with streptozotocin and with the development of assay methods for this antibiotic.

ASSAY METHODS

Disc-Plate Assay. A disc-plate method was used for assaying fermentation beers and preparations. Test plates were made as follows. One liter of nutrient agar was autoclaved for 15 minutes at 124 C., cooled to 50 C., and seeded with 2 ml. of an 18 hour shaken culture of *Proteus vulgaris* ATCC 8427 grown in nutrient broth. Five ml. of seeded agar was added to 22 × 100 mm. flat-bottom Petri dishes. After the agar had hardened, the test plates were stored at 4 C. until used.

A 40 µg./ml. standard solution was prepared in 0.1 M phosphate solution at pH 4. This solution was distributed in test tubes (5 ml. per tube), which were stored at -20 C. until used. On each assay day a tube was thawed, and standard solutions containing 40, 20, 10, 5, and 2.5 µg./ml. of streptozotocin were made, using the phosphate solution as a diluent. All five dilutions of the standard were individually spotted on 12.7 mm. filter paper discs (Schleicher and Schuell 740E) with 0.08 ml./disc. Eight plates, each containing all five standards, were run at the time of each assay.

All test samples were dissolved and/or diluted with the pH 4 phosphate solution, and 0.08 ml. was pipetted on one disc per plate on each of four plates.

All plates were incubated at 30 C. for 16 to 19 hours. The diameters of zones of inhibition were measured, and the zones from each sample or standard solution were averaged. The potencies of test solutions were determined from a standard curve plotted as logarithm dose versus zone diameter (fig. 1, curve A). The standard error of the method was estimated to be 20 per cent.

Whole Blood or Plasma. The relative instability of streptozotocin in solutions at the pH of blood and other tissues necessitated some modifications of the usual blood serum assay methods. Blood specimens had to be either assayed at the time of collection or the activity stabilized by lowering the pH of the solution immediately. Details of streptozotocin instability will be given in the section on antibacterial studies.

For the assay of streptozotocin in plasma, freshly drawn whole blood was oxalated with one drop of 20 per cent potassium oxalate per 5 ml. of blood. The blood was mixed, centrifuged as soon as possible, and 2 ml. of plasma was re-

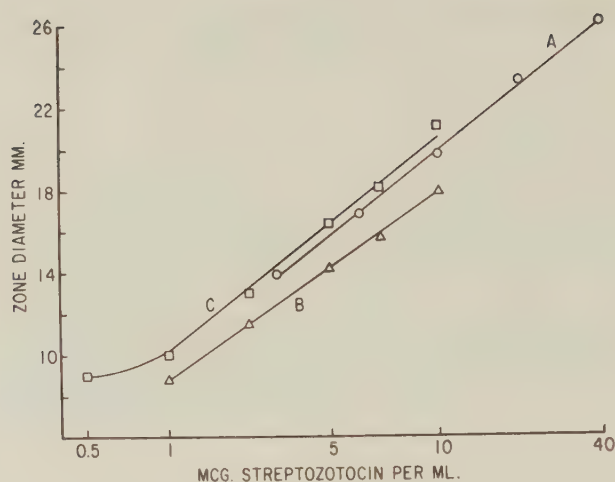


FIG. 1. Dose-response curves for streptozotocin. A, disc-plate method; B, plasma disc-plate method; C, plasma cup-plate method.

covered. One tenth ml. of 2 *N* hydrochloric acid was added, and the acidified plasma was stored at 4 C. until tested. The plasma was never stored for more than 72 hours before assay.

The test plates were made as follows. Dehydrated nutrient agar, 2.3 Gm., was added to 50 ml. of water. Fifty ml. of 0.1 *M* potassium phosphate buffered at pH 6 was added to a second flask. Both flasks were autoclaved for 15 minutes at 124 C., after which time the two solutions were combined. The medium was cooled to 50 C. and was inoculated with 0.2 ml. of a 24 hour shaken culture of *Proteus rettgeri* UC 399. Five ml. portions of the seeded agar were distributed in 22 × 100 mm. flat-bottom Petri dishes. The plates were stored at 4 C. until used.

Streptozotocin standard solutions were prepared in 0.1 *M* potassium dehydrogen phosphate solution at pH 4.5, at concentrations of 10, 6, 4, 2, 1, and 0.5 µg./ml. These solutions were stored at 4 C. and were used within one week after preparation.

The plasma and standard solutions were assayed by dipping ¼ inch discs (Schleicher and Schuell 740E) into the solutions and placing them on the seeded test plates. Four discs were dipped into each solution and were placed on each of four plates. The plates were incubated at 30 C. for 16 to 18 hours. The diameters of the zones of inhibition from each solution were averaged, and potencies of test

TABLE I

*Effect of pH on the Stability of Streptozotocin Solutions**

Buffer pH	Hours after addition of antibiotic to buffer							
	0	0.5	1.5	2	4	8	21	72
4	27	28	27	27	25	27	26	27
5	28	28	27	26	25	26	26	25
6	28	28	27	24	25	24	21	0
6.5	27	26	26	25	23	22	0	0
7	22	20	16	16	0	0	0	0
8	20	18	0	0	0	0	0	0

* The figures in the table are mm. zone sizes on agar plates seeded with *Proteus vulgaris*.

solutions were estimated from a standard curve plotted as logarithm dose versus diameter (fig. 1, curve B). The standard error of the method was estimated to be 18 per cent.

With the exception of the acidification step, the same procedure can be used to assay streptozotocin in whole blood. However, whole blood must be assayed immediately because of the instability of the antibiotic.

This assay method can be modified to a cylinder-plate method, in which two-layer test plates are made—a base layer consisting of 10 ml. of unseeded nutrient agar and a seeded layer of 4 ml., prepared as described in the disc-plate method. The dose-response curves using this method is given in figure 1, curve C.

STABILITY STUDIES

During early *in vitro* studies, it was apparent that streptozotocin was relatively unstable in fermentation beers. One of the first studies in the development of an assay method was to determine the *pH* of optimum stability. In this study, streptozotocin was incubated at 37 C. in phosphate buffers of *pH* 4 to 8, at a concentration of 40 µg./ml. Aliquots were removed at intervals, and 0.08 ml. was pipetted immediately onto a 12.7 mm. assay disc placed on an assay plate seeded with *P. vulgaris*. The plates were incubated at 30 C. for 16 hours, and the zones of inhibition were measured to the nearest millimeter. Table I indicates that degradation occurred rapidly at the higher *pH* values and that no detectable inactivation was evident at *pH* 4. The same results were obtained when nutrient broth or human serum replaced the buffer.

BACTERICIDAL STUDIES

The apparent inactivation of streptozotocin at *pH* 6 and 7, the physiological range, complicated *in vitro* end point determinations and turbidimetric assays. For example, a concentration of 50 µg./ml. of streptozotocin did not seem to inhibit growth of *P. vulgaris* in nutrient broth after 24 hours' incubation, while a response was obtained with 3 µg./ml. on seeded nutrient agar plates. The lack of activity in broth may be due to inactivation of streptozotocin at *pH* 7, with subsequent growth of the test organism upon prolonged incubation, and the presence of activity on agar may be due to bactericidal action of the antibiotic.

In order to illustrate this point, viable count studies were initiated. All viable counts were made using the Astell* vial method. One half ml. of each diluted suspension of organisms was mixed with 4.5 ml. nutrient agar at 48 C. in a vial. The vial was immediately attached to a spinning apparatus and the agar solidified with cold water jets. All vials were incubated at 37 C. for 16 to 24 hours and colonies counted with a magnifying glass.

In the first study, the effect of 10 µg./ml. of streptozotocin on viable count was investigated in nutrient broth at *pH* 6.8, since the drug appeared to be ineffective against *P. vulgaris* after 20 hours of incubation at 37 C. under these conditions when observed visually. One ml. of a 24 hour *P. vulgaris* culture was added to 9 ml.

* Astell Laboratory Service Co., Ltd., London, England.

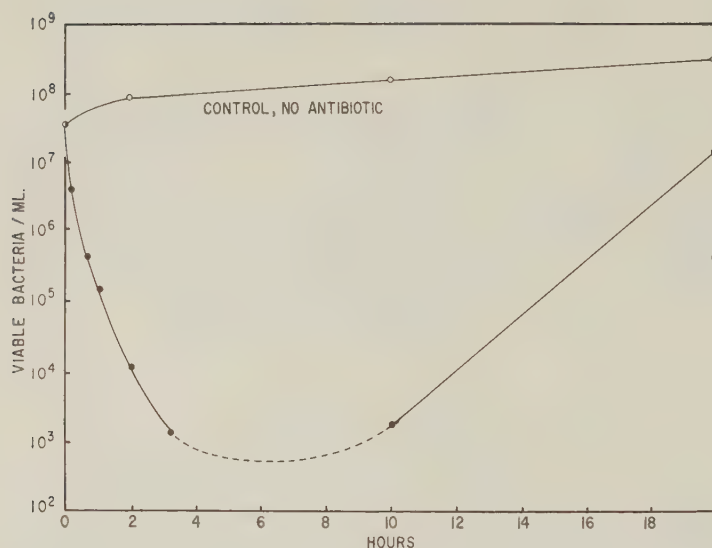


FIG. 2. The effect of streptozotocin on *Proteus vulgaris*.

of nutrient broth, and the seeded culture was incubated for one and one-half hours at 37 C., with vigorous shaking. The suspension was then divided into two aliquots, and streptozotocin was added to one to give a final concentration of 10 $\mu\text{g./ml.}$; an equal volume of sterile water was added to the other as a control. Both tubes were incubated at 37 C. and aliquots were counted periodically.

Figure 2 shows that streptozotocin was bactericidal during three hours of contact, although the culture resumed growth after 10 hours. The 10 hour sample contained less than 2.5 $\mu\text{g./ml.}$ streptozotocin.

The second experiment (fig. 3) showed that a culture of *P. vulgaris* could be sterilized with streptozotocin. In this study, a 24 hour culture was diluted 800,000 fold with nutrient broth, and streptozotocin was added to give a final concentration of 4 $\mu\text{g./ml.}$ The culture was incubated at 37 C. for 18 hours, after which time

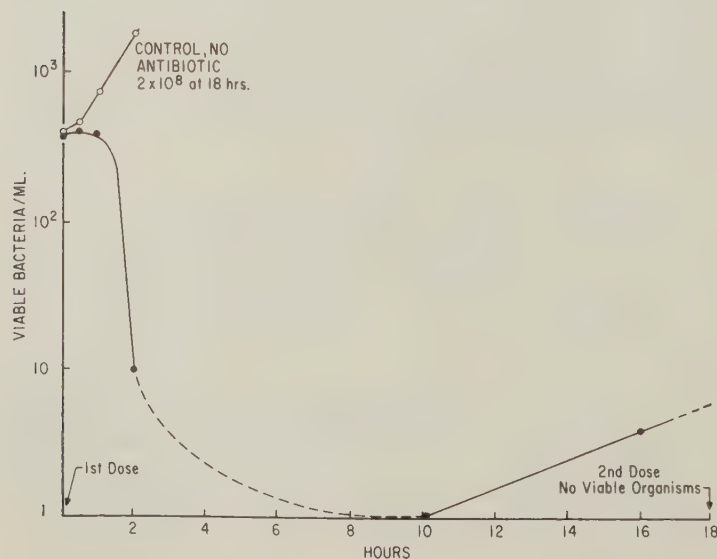
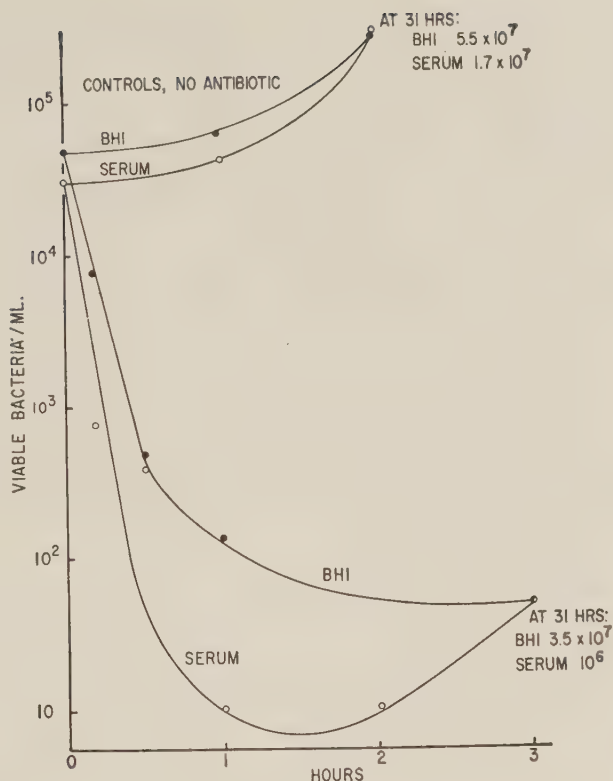


FIG. 3. The effect of two 4 $\mu\text{g./ml.}$ doses of streptozotocin on survival of *Proteus vulgaris*.

FIG. 4. The effects of 20 $\mu\text{g./ml.}$ streptozotocin on growth of *Staphylococcus aureus* in brain-heart broth and in human serum.



the antibiotic was again added. No subsequent growth was observed. Transfers into fresh medium indicated that the culture had been sterilized.

In the third experiment, indications of serum binding were sought. In this study, a 24 hour culture of *Staphylococcus aureus* FDA-209P was diluted 200-fold, and 0.2 ml. volumes of the diluted suspension were added to 20 ml. each of pooled human serum and to brain-heart infusion broth at pH 7.4. Both tubes were incubated for one and one-half hours at 37 C. and then divided into two 10 ml. fractions. Streptozotocin was added to one fraction, while sterile water was added to the other (controls). All four tubes were then incubated without shaking in a water bath at 37 C. Viable counts were made at the indicated times. Figure 4 indicates that the antibiotic was at least as effective as a bactericidal agent in human serum as it was in brain-heart medium. It is assumed that if serum binding of streptozotocin occurred, the antibiotic would appear less effective in the serum than in the medium.

DISCUSSION

The instability of streptozotocin in broth (fig. 2) could explain the apparent low order of activity of the antibiotic in broth. However, in the disc-plate assay, activity was observed with solutions containing 2.5 $\mu\text{g./ml.}$ This response, after incubation periods of sufficient time to inactivate the streptozotocin, was probably the result of the bactericidal activity around the disc before antibiotic inactivation occurred.

The diffusion rate of streptozotocin in agar apparently is not affected by differences in salt concentration in the agar or test solutions as are other antibiotics, e.g., neomycin⁵ and streptomycin.⁶ Standard dose-response curves are identical in the presence or absence of salts in either the nutrient agar assay medium or the standard solutions. In fact, standard curves with streptozotocin in pH 4 or 6 buffers in plasma and in whole blood are approximately the same.

The possibility of assaying whole blood for streptozotocin concentration has the advantage of being able to omit the steps of separation of plasma and acidification, but has the disadvantage of the necessity for immediate assay. This method would not be convenient in a blood level versus time study.

SUMMARY

Microbiological methods for the assay of streptozotocin are described. The test organisms used were *Proteus vulgaris* for the disc-plate assay and *Proteus rettgeri* for the blood plasma assay.

In vitro studies with streptozotocin showed a good dose-response on nutrient agar plates but little or no activity against the same test organisms in nutrient broth after 18 hours' incubation at 37 C. The relative instability and good bactericidal activity of streptozotocin were demonstrated, which explains the phenomenon of activity in the plate assay and lack of activity in the broth assay.

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Streptozotocin, a New Antibiotic. In Vitro and In Vivo Evaluation

CHARLES LEWIS AND ARTHUR R. BARBIERS

Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

A new antibiotic, streptozotocin, has been isolated in these laboratories from the fermentation broth of a strain of *Streptomyces achromogenes* var. 128. Preliminary observations¹ during the antibiotic's development indicated that it possessed good antibacterial activity against both gram-positive and gram-negative bacteria. Studies on the methods of its isolation, as well as some of its chemical, physical, and biological properties, are reported in the accompanying papers.²⁻⁴ It is the purpose of the present paper to report the results of antibacterial studies in which streptozotocin was used.

MATERIALS AND METHODS

In Vitro. The sensitivity of bacteria to streptozotocin was determined by a standard twofold tube dilution method⁵ in brain-heart infusion broth (Difco) adjusted to a pH of 6.4. Streptozotocin was dissolved in pH 6.0 phosphate buffer. End point determinations were read by visual inspection at eight hours and verified by establishing the culture's optical density in an automatic recording nephelometer.⁶ The effect of human and rabbit serum, human urine, and hydrogen ion concentration on the inhibitory properties of streptozotocin was determined in brain-heart infusion broth (Difco).

In Vivo. Male white mice (CF-1), 18 to 20 Gm. (unless otherwise specified), were selected at random from animal pools for use in the animal protection tests. Techniques for testing compounds in infected mice have been previously described.⁷ Chronic kidney infections⁸ in mice were produced by the intravenous injection of 0.2 ml. containing 10^6 to 10^7 cells of an 18 hour broth culture of the Smith strain of *Staphylococcus aureus*.

Freshly prepared stock solutions of the antibiotic were prepared at the beginning of each experiment in phosphate buffer adjusted to pH 6.0. The antibiotic concentration was such that the desired dose was contained in 0.2 ml. when administered subcutaneously or in 0.5 ml. when administered by gastric intubation. In the mouse protection test, the infecting dose of bacteria and the drug were administered so that the drug was given immediately following the bacterial challenge. Therapy was continued at daily intervals for four days and the mice observed for an additional three days. Evaluation of antibiotic activity was based at the end of seven days on its median protective dose (CD_{50}) in groups of 10 animals challenged with 100 LD_{50} of organisms. In experiments concerned with chronic kidney infections, streptozotocin was administered subcutaneously for 13 days. On the fourteenth day postinfection, the animals were sacrificed, and the kidneys from each mouse were removed and ground in Pyrex tissue grinders containing 4 ml. brain-heart infusion broth. The number of viable cells was determined by plating 0.1 ml. of the organ

TABLE I
The Antibacterial Spectrum of Streptozotocin

Organism	Strain (Upjohn no.)	Minimal inhibitory conc., µg./ml.
<i>Staphylococcus aureus</i>	76	0.39
	317	3.1
	391	1.5
	488	1.5
	595	0.78
	599	0.78
	601	0.78
	607	0.78
	703	0.78
	704	3.1
	738	0.78
	739	6.2
	740	6.2
	749	0.78
<i>Diplococcus pneumoniae</i>	41	1.5
	42	>100
	43	3.1
	44	6.2
	190	1.5
<i>Streptococcus hemolyticus</i>	752	100
	753	50
	754	<6
	755	<6
	756	100
	757	100
	758	100
	759	100
	778	>100
	779	<12
	780	100
	769	25
<i>Streptococcus faecalis</i>	152	25
	157	12.5
	700	6.2
	701	3.1
<i>Alcaligenes faecalis</i>	4	>100
	526	1.5
<i>Listeria monocytogenes</i>	223	>100
<i>Escherichia coli</i>	311	0.39
	422	12.5
	527	12.5
	557	100
<i>Aerobacter aerogenes</i>	437	100
	438	50
	441	100
	444	3.1
	445	1.5
<i>Proteus morganii</i>	340	0.39
	341	0.39
	346	0.78
<i>Proteus rettgeri</i>	339	0.02
	342	12.5
	344	0.04
<i>Proteus vulgaris</i>	232	0.78
	338	6.2
	343	0.39
<i>Pseudomonas aeruginosa</i>	347	>100
	348	>100
	350	>100
	351	>100
	400	>100

Table I Continued on Page 249

TABLE I (Continued)
The Antibacterial Spectrum of Streptozotocin

Organism	Strain (Upjohn no.)	Minimal inhibitory conc., µg./ml.
<i>Pasteurella multocida</i>	264	25
<i>Salmonella enteritidis</i>	113	25
	114	25
<i>Salmonella gallinarum</i>	145	0.78
	265	6.2
<i>Salmonella hirschfeldii</i>	259	0.78
<i>Salmonella paratyphi</i> A	260	12.5
<i>Salmonella paratyphi</i> B	262	0.09
<i>Salmonella typhosa</i>	105	3.1
	215	3.1
	643	1.5
<i>Salmonella pullorum</i>	267	0.78
<i>Shigella dysenteriae</i>	139	> 100
<i>Shigella flexneri</i>	143	0.78
	144	0.78
<i>Shigella paradysenteriae</i>	134	6.2
<i>Klebsiella pneumoniae</i>	58	3.1
	272	> 100
	277	> 100
<i>Neisseria catarrhalis</i> *†	87	> 100
	88	> 100
	89	> 100
<i>Neisseria meningitidis</i> *	548	> 100
<i>Neisseria gonorrhoeae</i> ‡	794	> 100
<i>Hemophilus pertussis</i> §	777	> 100
<i>Clostridium botulinum</i>	244	100
<i>Clostridium perfringens</i>	247	100
<i>Clostridium sporogenes</i>	72	100
<i>Clostridium tetani</i>	680	> 100

* Tested in dextrose-starch agar.

† The *N. catarrhalis* cultures gave negative oxidase tests, indicating they may not be true *Neisseria*.

‡ Chocolate agar (oxidase positive).

§ Blood agar.

homogenate diluted in tenfold increments on the surface of agar plates. After incubation, suitable plates were counted and the number of bacteria expressed as the logarithm of the colony count per pair of kidneys. Evaluation of the antibiotic was based on the reduction of bacteria in the kidneys from treated mice as compared to that of kidneys from untreated control animals.

TABLE II
Effect of Human and Rabbit Serum on the Activity of Streptozotocin*

Source of serum	Serum concentrations, per cent							
	<i>Staph. aureus</i> UC-76				<i>P. vulgaris</i> UC-232			
	50	25	10	0	50	25	10	0
Human	0.78	0.39	0.39	0.19	0.78	0.78	0.78	0.78
Rabbit	0.78	0.78	0.39	0.39	1.5	1.5	1.5	1.5

* End point as µg./ml. required for complete inhibition of growth at seven hours.

TABLE III

*The Effect of Urine on the Activity of Streptozotocin**

Concentration of urine, %	Test organisms			
	<i>Staph. aureus</i> UC-76		<i>P. vulgaris</i> UC-232	
	7 hours	24 hours	7 hours	24 hours
50	0.39	>100	0.78	25
25	0.39	>100	0.78	25
0	0.39	>100	0.78	50

* End point as $\mu\text{g./ml.}$ for complete inhibition.

RESULTS

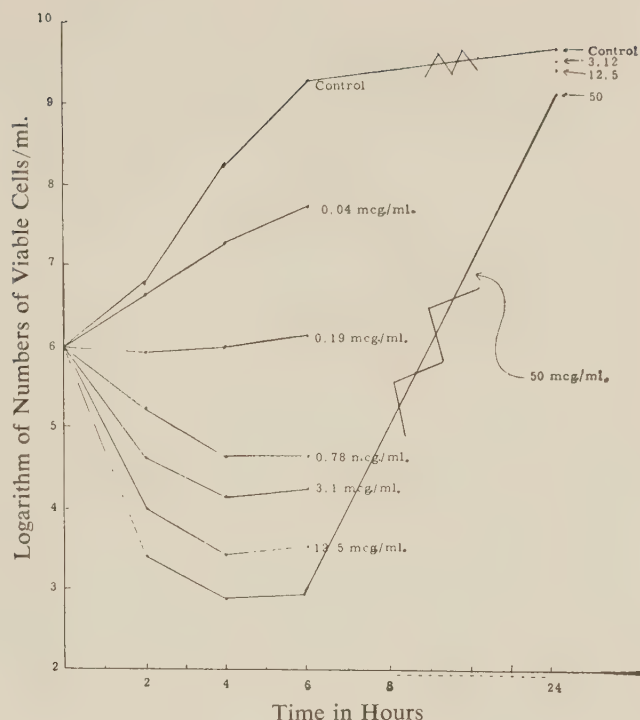
In Vitro. Streptozotocin possessed good activity against micrococci, diplococci, and enterococci, as well as *Pasteurella*, *Salmonella*, and *Proteus* species. It was also active against species of *Alcaligenes*, *Streptococcus*, *Aerobacter*, and coliform microorganisms, but wide variations in sensitivity were observed. At the highest level tested (100 $\mu\text{g./ml.}$), streptozotocin did not inhibit the growth of *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, or strains of the genera *Neisseria*, *Clostridium*, or *Hemophilus* (table I). The end points listed in table I were determined after eight hours' incubation. The validity of using eight hour end points was verified by first establishing end points for chloramphenicol, tetracycline, novobiocin, and penicillin against *Staph. aureus*. The activity of the antibiotics at eight hours was identical to that observed at 24 hours. The shorter period of time for end point determinations was used in these experiments, since streptozotocin was unstable in common laboratory media over the 24 hour period usually used for observation.

Experiments designed to test the effect of human and rabbit serum on the in vitro activity of streptozotocin demonstrated that concentrations of either human or rabbit serum as high as 50 per cent had no effect on the in vitro activity of the antibiotic against *Staph. aureus* or *Proteus vulgaris* (table II). Concentrations of pooled human urine as high as 50 per cent had no effect on the in vitro activity of the antibiotic (table III).

The effect of varying hydrogen ion concentrations on the in vitro end points (eight hours) of streptozotocin was determined by the twofold dilution technique, using brain-heart infusion broth adjusted to the desired hydrogen ion concentration. *Staph. aureus* was used as the test organism. The results demonstrated that as the hydrogen-ion concentration was decreased, the activity of streptozotocin decreased, although activity against *Staph. aureus* was still present at pH 8.0. The poor bacterial growth at lower pH levels made it difficult to determine if increased activity was due to the antibiotic present or to the lack of growth. However, the inhibitory effect on the growth of *Staph. aureus* to streptozotocin in media at pH 6.4 to 6.7, as compared to *Staph. aureus* growth in nonantibiotic supplemented control media, demonstrated that the optimal hydrogen ion concentration for streptozotocin activity was below pH 7.0.

The in vitro bactericidal properties of streptozotocin were investigated by using

FIG. 1. Effect of streptozotocin on the viable cell counts of *Staph. aureus*.



Staph. aureus as the test organism, and the tests were carried out in brain-heart infusion broth adjusted to pH 6.4. Results (fig. 1) reveal that as little as 0.04 $\mu\text{g./ml.}$ of the antibiotic had some effect on the growth of *Staph. aureus*, but that concentrations as high as 50 $\mu\text{g./ml.}$ failed to kill all the cells present in the inoculum. Results of a similar experiment in which an analysis was made of the cell population at various times during the growth phase of a culture are presented in table IV. The data reveal that resistant cells were present after a four hour incu-

TABLE IV
*Effect on the Viable Count of Staph. aureus in Media
Supplemented with Streptozotocin*

Hour of Sampling	Control medium* (unsupplemented)	Viable count	
		Exposed to 100 $\mu\text{g./ml.}$ streptozotocin†	
		Unsupplemented agar‡	Supplemented agar§
0	4.25×10^5	5.0×10^5	—
4	6.05×10^7	40	22
8	9.00×10^9	320	389
24	2.95×10^9	9.8×10^8	9.4×10^8

* Control culture grown in brain-heart infusion broth.

† *Staph. aureus* grown in brain-heart infusion broth supplemented with 100 $\mu\text{g./ml.}$ streptozotocin.

‡ Viable count as determined on brain-heart infusion agar.

§ Viable count as determined on brain-heart infusion agar supplemented with 100 $\mu\text{g./ml.}$ streptozotocin.

TABLE V

Effect on the Growth Curve of Staph. aureus of Adding Fresh Streptozotocin at Varying Times to the Culture Medium

Fresh drug added at	Lag phase in hours, $\mu\text{g.}/\text{ml.}$				
	100	50	10	1	Control
0 hours	16	14	12	10	2
0 hours and 2 hours	18	14	12	10	
0 hours and 4 hours	18	14	12	10	
0 hours and 6 hours	18	14	12	10	
0 hours and 2, 4, 6, hours	20	18	12	12	

bation period in the presence of 100 $\mu\text{g.}/\text{ml.}$, and that after a 24 hour incubation period in the presence of the drug, all cells were resistant to streptozotocin. The inoculum, consisting of 4.2×10^5 cells per ml., contained no cells resistant to 100 $\mu\text{g.}/\text{ml.}$

In order to eliminate the possibility of drug breakdown, streptozotocin was added at 0, 2, 4, and 6 hours to a culture of *Staph. aureus* containing an initial population of 1×10^6 cells per ml. The results of this experiment (table V) demonstrate that 100 $\mu\text{g.}/\text{ml.}$ of drug added every two hours over a six hour period did not sterilize the culture but merely caused some increase in the lag phase of the organism. In this experiment, turbidity measurements were made on the cultures every two hours over a 24 hour period. At the end of that time, these cultures were used as a source of inoculum for additional end point studies, which revealed that strepto-

TABLE VI

Effect of Streptozotocin on Staphylococci Having Varied Resistance Patterns

Strain no.	Resistance pattern	End point, $\mu\text{g.}/\text{ml.}$
Clinical Isolate		
488	Penicillin, chloramphenicol, and tetracycline	1.5
391	Penicillin, chloramphenicol, streptomycin, and tetracycline	1.5
317	Erythromycin, penicillin, streptomycin, and tetracycline	3.1
740	Penicillin and tetracycline; phage type 80/81	6.2
739	Penicillin, tetracycline and oleandomycin; phage type 80/81	6.2
738	Penicillin, tetracycline, and erythromycin; phage type 80/81	.78
749	Penicillin, chloramphenicol, erythromycin, tetracycline, and streptomycin	.78
76	Sensitive to antibiotics; phage type 44a	.39
Laboratory Strain		
607	Novobiocin	.78
601	Neomycin C	.78
704	Erythromycin-tetracycline	3.1
599	Neomycin A	.78
703	Erythromycin	.78
595	Celesticetin	.78

TABLE VII

*Activity of Streptozotocin in Experimentally Infected Animals**

Organism	Challenge LD ₅₀ †	Median protective dose, CD ₅₀ ‡	
		Subcutaneous	Oral
<i>Staph. aureus</i> 284	57	5.2 (3.8–6.7)	6.2 (4.3–8.1)
<i>Staph. aureus</i> 284 SR	230	1.6 (1.2–2.0)	4.3 (2.8–5.8)
<i>Str. hemolyticus</i> 203	45	80	400
<i>Str. hemolyticus</i> 26	81	36 (26–46)	243 (128–350)
<i>Str. viridans</i> UC 871	32	12.7 (10.5–14.9)	116 (87–145)
<i>D. pneumoniae</i> I (Felton)	316	80 (47–113)	320
<i>D. pneumoniae</i> III	263	23.5 (16.6–30.4)	320
<i>P. multocida</i> 449	151	1.6 (1.2–2.0)	4.0 (2.9–5.1)
<i>P. multocida</i> UC 802	1000	8 (6.2–9.8)	20 (18–22)
<i>Sal. paratyphi</i> B 4	224	13.3 (11.0–15.6)	22 (18.2–25.8)
<i>Sal. typhimurium</i> SB 682	80	28 (15–41)	121 (52–170)
<i>K. pneumoniae</i> A–D	37	24.4 (17.5–31.3)	160
<i>E. coli</i> UC 311	63	80	133 (85–181)
<i>P. vulgaris</i> 347	347	1.2 (0.7–1.7)	2.9 (2.1–3.6)
<i>Ps. aeruginosa</i> YED	260	80	400
<i>Sal. gallinarum</i> 1292	—	—	400

* Leghorn 4 day chicks were used in the *Sal. gallinarum* infection. In all others, CF-1 mice weighing 18 to 20 Gm. were used.

† Median lethal dose/animal, mg./Kg.

‡ As mg./Kg./day with 95 per cent confidence limits.

zotocin failed to prevent outgrowth of the inoculum. This latter experiment enabled us to conclude again that *Staph. aureus* had rapidly become resistant to streptozotocin.

Attempts to demonstrate cross resistance of streptozotocin with any of the commonly used antibiotics were negative. The results in table VI are from experiments in which a series of both clinical isolates with varying antibiotic resistance patterns and laboratory developed resistant strains were tested.

In Vivo. Streptozotocin was highly effective in protecting mice infected with either gram-positive or gram-negative organisms. As can be seen in the data (table VII), streptozotocin was most active against *Staph. aureus*, *Pasteurella multocida*, and *P. vulgaris*, with the median protective dose (CD₅₀) ranging from 1.2 to 5.2 mg./Kg./day subcutaneously and 2.9 to 6.2 mg./Kg./day orally. Somewhat higher

TABLE VIII

*Effect of Streptozotocin on Chronic Staph. aureus Infections in Mice**

Drug level, mg./Kg./day†	Mortality ratio	Bacterial count/Gm. of kidney
12.5	2/10	2.8 x 10 ⁷
25	0/10	2.9 x 10 ⁷
50	0/10	1.5 x 10 ⁷
100	0/10	0.7 x 10 ⁷
200	0/10	0
0	0/10	4 x 10 ⁷

* Mice infected intravenously with 0.2 ml. of 1:5 dilution of 18 hour broth culture of Smith strain.

† Drug administered orally for 14 days.

CD₅₀ values were obtained against *Streptococcus hemolyticus*, *Streptococcus viridans*, *Diplococcus pneumoniae*, *Salmonella paratyphi* B, *Salmonella typhimurium*, and *K. pneumoniae*. No activity could be demonstrated in infections due to *P. aeruginosa*, *Salmonella gallinarum*, or *Mycobacterium tuberculosis*.

Data presented in table VIII demonstrate that streptozotocin possesses some activity in chronic *Staph. aureus* infections patterned after those described by Smith and Dubos.⁸ Groups of infected mice treated orally at 100 and 200 mg./Kg./day demonstrate some response to the antibiotic, although only the top level tested (200 mg./Kg./day) sterilized the kidneys.

SUMMARY

Data have been presented that illustrate good in vitro and in vivo activity of streptozotocin against a wide variety of both gram-positive and gram-negative organisms.

A streptomycin-type resistance was developed in vitro. There was no evidence of cross resistance either with laboratory developed strains or with resistant cultures from clinical sources.

Streptozotocin was effective over a wide pH range, and its activity was not altered by the presence of human or rabbit blood serum nor human urine.

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Bacterial Resistance to Streptozotocin

L. J. HANKA AND W. T. SOKOLSKI

Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Streptozotocin is a new antibiotic that has broad in vitro activity against many gram-positive and gram-negative bacteria and has in vivo activity against most of these organisms in mice.¹⁻⁴ It is rather unstable in solution at physiological pH and temperature. In phosphate buffer of pH 4 and at 4 C., however, it can be stored for several weeks without any significant loss of potency.

The purpose of the present investigation was to obtain the following information concerning bacterial resistance to this antibiotic: (1) The nature of resistance development; (2) the frequency of occurrence of spontaneously resistant mutants; (3) the rate of back-mutations toward sensitivity; (4) cross resistance with other antibiotics; and (5) mechanism responsible for bacterial resistance to streptozotocin. The entire study was done on the premise of spontaneous mutations, a theory that has been well established by other workers.^{5,6} All investigations were carried out with *Staphylococcus aureus*, strains FDA-209P and TUC-284 and *Proteus vulgaris* strain ATCC 8427.

METHODS

Determination of the Type of Resistance of Staph. aureus and P. vulgaris to Streptozotocin. Tubes containing 5 ml. of brain-heart infusion broth (pH = 6.8) were supplemented with 5 µg./ml. of streptozotocin and inoculated with one drop of a 5 hour culture of the test organism. The tubes were incubated overnight at 37 C. and plated on nutrient agar containing 5 µg./ml. of the antibiotic. This procedure should have resulted in the isolation of colonies resistant to at least 5 µg./ml. of streptozotocin if resistant bacteria were present.

To test the level of bacterial resistance, two experiments were carried out: (1) Forty colonies from two different plates were each dispersed in 2 ml. of sterile water and one drop of each suspension was used as inoculum for tubes containing brain-heart infusion broth with streptozotocin at 0, 5, 20, 50, and 100 µg./ml. The tubes were incubated for 7 hours at 37 C. and growth was measured turbidimetrically. (2) Two of the plates were replicate plated⁷ on nutrient agar containing increasing concentrations of streptozotocin at 0, 5, 20, 50, and 100 µg./ml. Control plates were prepared using the sensitive parent strain and replicate plated the same way as the resistant colonies. The plates were incubated overnight at 37 C. and the colonies counted.

Determination of the Frequency of Occurrence of Resistant Mutants in the Sensitive Parent Population. A 24 hour brain-heart infusion culture of the two strains of *Staph. aureus* (single colony isolates) was plated out on nutrient agar for a total viable count and on nutrient agar containing 20 µg./ml. of streptozotocin for the isolation of resistant cells. The same method was used for estimation of the rate of occurrence of resistant cells in a sensitive population of *P. vulgaris*, except with

10 µg./ml. streptozotocin instead of 20 µg./ml. Both plates were incubated overnight at 37 C. and the colonies were counted.

To verify the estimated frequency of appearance of streptozotocin-resistant mutants, the following experiment was designed: Tubes containing brain-heart infusion broth with 100 µg./ml. of streptozotocin at pH 6.8 were inoculated with two different levels from the sensitive parent strains of *Staph. aureus*. Five tubes were inoculated with approximately 1×10^7 cells and five tubes were inoculated with approximately 1×10^3 cells/tube. The controls were inoculated into tubes containing no antibiotic, and all tubes were incubated for 20 hours at 37 C. If the estimated frequency was correct, there should be no growth in tubes where small inoculum was used, but there should be growth in tubes where the inoculum was large enough to contain some resistant cells. The same experiment was run with *P. vulgaris*.

Rate of Back-Mutations to Streptozotocin Sensitivity. Two resistant strains of *Staph. aureus* were subcultured once or twice daily into brain-heart infusion broth. To determine whether there was any decrease in the proportion of resistant cells in the population, the culture was repeatedly plated out simultaneously on nutrient agar and nutrient agar containing 20 µg./ml. of streptozotocin. Plates were incubated overnight at 37 C. and colonies counted. Both strains were carried for 50 consecutive transfers in the absence of streptozotocin.

Cross-Resistance Study. The cross-resistance pattern of streptozotocin was determined in two experiments. In the first experiment several mutant microorganisms, resistant to the common antibiotics, were tested against streptozotocin. In the second experiment, a strain of *Staph. aureus* that was developed resistant to 100 µg./ml. of streptozotocin was tested against several of the common antibiotics. The sensitivities were measured by a disc plate method and zones of inhibition were measured following 20 hours of incubation at 37 C.

Physiological Background of Bacterial Resistance to Streptozotocin. A study was made to obtain information on the fate of streptozotocin in cultures of resistant and sensitive strains of *Staph. aureus*. Two tubes containing 10 ml. of nutrient broth and 100 µg./ml. of streptozotocin were inoculated with one drop of a 20 hour culture of the sensitive parent strain and a resistant strain respectively. Both tubes were incubated at 37 C. and samples of the cultures were taken at several time intervals and the levels of streptozotocin estimated by microbiological assay.³ Also, the optical density of both tubes was measured at 0.5, 2, 4, and 7 hours (Bausch and Lomb spectrophotometer, 650 mµ).

At the end of the growth period, samples from both tubes were tested for possible bioconversion products by paper chromatography using the following six systems: *n*-butanol:water, 84:16 by volume; *n*-butanol:water, 84:16 plus 0.25 per cent (w/v) *p*-toluenesulfonic acid; *n*-butanol:acetic acid:water, 2:1:1 by volume; *n*-butanol:water, 84:16, 2 ml. piperidine added to 98 ml. of butanol-water mixture; water:*n*-butanol, 96:4 (v/v); and water:*n*-butanol, 96:4 plus 0.25 per cent *p*-toluenesulfonic acid.

In another study, the sensitive strains of *Staph. aureus* FDA-209P and TUC-284 were grown overnight in brain-heart infusion broth. The streptozotocin-resistant mutants of both strains were grown overnight in medium containing 100 µg./ml. of the antibiotic. Streptomycin agar (Difco) was seeded with the two sensitive strains at the rate of 0.2 ml. of culture per 100 ml. of agar. Each of the two seeded

TABLE I
Number of Colonies on Replicate Plates of *Staph. aureus*
Containing Increasing Levels of Streptozotocin

Strain	Master plate	Levels of streptozotocin on replicate plates, $\mu\text{g./ml.}$				
		0	5	20	50	100
FDA-209 resistant to 5 $\mu\text{g./ml.}$	29	>23	20	19	18	16
FDA-209 sensitive	110	110	0	0	0	0
TUC-284 resistant to 5 $\mu\text{g./ml.}$	25	25	25	20	19	18
TUC-284 sensitive	300	300	0	0	0	0

agars were divided into two aliquots and streptozotocin was added to one for a final concentration of 10 $\mu\text{g./ml.}$ and pipetted to Petri dishes with 10 ml. per dish. A heavy streak of each resistant culture was made across each plate containing the parent of that resistant strain and the plates were incubated overnight at 37 C.

It was expected that nothing other than the resistant bacteria in surface streak would grow on the plates containing 10 $\mu\text{g./ml.}$ of streptozotocin and the sensitive parent. If the mutant strain was resistant because of production of an extracellular enzyme which destroys streptozotocin (comparable to penicillinase) there should be some growth of the sensitive strain in close proximity to the resistant growth.

RESULTS AND DISCUSSION

Determination of the Type of Resistance of Staph. aureus and P. vulgaris to Streptozotocin. The progeny of 40 resistant colonies of *Staph. aureus* isolated in media containing 5 $\mu\text{g./ml.}$ of streptozotocin were able to grow in the presence of 0, 5, and 20 $\mu\text{g./ml.}$ of streptozotocin at approximately equal rates. The growth was somewhat less abundant in tubes containing 50 $\mu\text{g./ml.}$ and substantially lower in the presence of 100 $\mu\text{g./ml.}$ However, there was only one out of 40 tubes that had no growth in the presence of 100 $\mu\text{g./ml.}$ after eight hours at 37 C. None of the progeny of the sensitive colony controls grew in the tube with antibiotic.

The replicate plate technique revealed that most of the colonies resistant to 5 $\mu\text{g./ml.}$ were also resistant to higher levels of the antibiotic. Detailed results are presented in table I. The results of the experiment with *P. vulgaris* are presented in table II. It appears that with *Staph. aureus* the resistance toward streptozotocin

TABLE II
Number of Colonies on Replicate Plates of *P. vulgaris*
Containing Increasing Levels of Streptozotocin

Strain	Master plate	Levels of streptozotocin on replicate plates, $\mu\text{g./ml.}$				
		0	5	20	50	100
Sensitive parent	42	>32	0	0	0	0
Sensitive parent	41	>30	0	1	0	0
Resistant to 10 $\mu\text{g./ml.}$	55	>50	>45	27	0	0
	115	>45	>45	>45	4	3

TABLE III

Number of Naturally Resistant Colonies in 2 Sensitive Strains of Staph. aureus on Plates Containing 20 µg./ml. Streptozotocin in the Agar

Strain*	Counts									
FDA-209	116	112	105	113	131	134	129	120	141	108
(dilution 1/200)	109	128	219	136	155	125	113	127	128	126
Total resistant count: 2.5×10^4 cells/ml.										
TUC-284	225	226	200	219	241	203	230	245	234	217
(dilution 1/20)	210	200	222	218	241	210	175	211	201	187
Total resistant count: 4.32×10^3 cells/ml.										

* The total viable count for strain 209 was 2.84×10^9 and 4.00×10^9 for strain 284. The proportion of resistant mutants in the sensitive parent population was 1 in 1.14×10^5 for strain FDA-209 and 1 in 9.25×10^5 for strain TUC-284.

was overwhelmingly developed to the highest levels in a single step. However, with *P. vulgaris* the resistance development appeared to be a gradual process. This observation was further supported by the inability to isolate any resistant mutants of *P. vulgaris* with first contact on plates containing 100 µg./ml.

Determination of the Frequency of Occurrence of Resistant Mutants in the Sensitive Parent Population. Table III presents the results of differential plating and gives the proportion of naturally resistant cells in the sensitive parent population of two strains of *Staph. aureus*. Similar data are also given for *P. vulgaris*.

The numbers of colonies of *P. vulgaris* isolated on plates containing 10 µg./ml. of streptozotocin were: 20, 21, 18, 13, 24, 18, 21, 18, 21, 19, 23, 20, 19, 25, 22, 29, 29, 22, 21, and 22. The number of resistant cells was 4.25×10^3 and the total viable count was 1.91×10^9 cells per ml. The frequency of occurrence of resistant cells in the sensitive parent population was one in 4.5×10^5 .

The estimated mutation frequencies for both *Staph. aureus* and *P. vulgaris* were verified by experiments where no resistant growth was produced in broth containing bactericidal levels of streptozotocin and 1×10^3 sensitive cells. On the other hand, with a population of 1×10^7 cells, resistant progeny was found in all tubes. During these experiments, the occurrence of "persisters"⁸ was noted. These were sensitive cells which survived the effect of normally bactericidal doses of the antibiotic.

Rate of Back-Mutations to Streptozotocin Sensitivity. After 50 consecutive transfers of two resistant strains of *Staph. aureus* in media containing no antibiotic, prac-

TABLE IV

*Viable Counts of 2 Streptozotocin-Resistant Strains of Staph. aureus in the Presence and Absence of Streptozotocin (Incubated for 18 hours at 37 C.)**

Strain	Medium	Viable count
FDA-209	Nutrient agar	0.99×10^9
FDA-209	Nutrient agar plus 100 µg./ml. streptozotocin	0.86×10^9
TUC-284	Nutrient agar	1.02×10^9
TUC-284	Nutrient agar plus 100 µg./ml. streptozotocin	1.03×10^9

* Plated after 50 consecutive transfers in the absence of streptozotocin.

TABLE V

Zones of Inhibition of Several Antibiotic-Resistant Strains of Staph. aureus by Streptozotocin (6.5 mm. discs used)*

Strain	Resistant to	Antibiotic applied	Conc. of antibiotic, $\mu\text{g.}/\text{disc}$	Zone, mm.
UC 488	Penicillin	Penicillin	10	0
UC 488	Penicillin	Streptozotocin	10	17
UC 571	Erythromycin	Erythromycin	15	0
UC 571	Erythromycin	Streptozotocin	10	15
UC 599	Neamine, neomycin	Neamine	10	0
UC 599	Neamine, neomycin	Neomycin B	10	0
UC 599	Neamine, neomycin	Streptozotocin	10	15
UC 595	Celesticetin	Celesticetin	10	0
UC 595	Celesticetin	Streptozotocin	10	15

* Zone size expressed in mm. around 6.5 mm. disc.

TABLE VI

Growth Inhibition of Streptozotocin-Resistant Mutant and of the Sensitive Parent Strain of Staph. aureus by Antibiotics (13 mm. discs, 0.08 ml. of antibiotic solution per disc)

Antibiotic	Concentration, $\mu\text{g.}/\text{ml.}$	<i>Staph. aureus</i> parent	<i>Staph. aureus</i> resistant to streptozotocin
Streptozotocin	100	>25	0
	50	>25	0
	25	>25	0
	12.5	25	0
Neomycin	20	19	19
	10	18	17
	5	16.5	16.5
	2.5	15.5	15.5
Polymyxin	1000	20	18.5
	500	17	16
	250	Trace	Trace
	125	0	0
Tetracycline	25	>25	>25
	12.5	>25	>25
	6.3	>25	24.5
	3.1	25	24
Kanamycin	50	20.5	18.5
	25	18	16.5
	12.5	16.5	15.5
	6.3	15	Trace
Penicillin	25	>25	>25
	12.5	>25	>25
	6.3	>25	>25
	3.1	>25	>25
Novobiocin	25	>25	>25
	12.5	>25	>25
	6.3	24	>25
	3.1	23	24
Erythromycin	50	25	20
	25	22.5	19
	12.5	20	18
	6.3	18.5	16.5
Chloramphenicol	50	22.5	21.5
	25	18	17
	12.5	15	15
	6.3	Trace	0
Carbomycin	50	18	17
	25	0	0

TABLE VII

Rate of Disappearance of Streptozotocin in Growing Cultures of Sensitive and Resistant Strains of Staph. aureus

Incubation time, hr.	Optical density		Concentration of streptozotocin, $\mu\text{g./ml.}$	
	Sensitive parent	Resistant mutant	Sensitive parent	Resistant mutant
0			100.8	95.2
1/2	.25	.38	75.0	96.3
1			72.8	77.3
2	.25	.55	59.9	66.1
4	.25	.75	43.7	58.8
7	.25	.85	19.6	39.2

tically the entire population was resistant to 100 $\mu\text{g./ml.}$ of streptozotocin. The results of the differential count are presented in table IV. It appeared that the resistance of *Staph. aureus* toward streptozotocin is retained tenaciously.

Cross-Resistance Study. Table V shows the inhibition zone sizes from streptozotocin with several antibiotic-resistant strains of *Staph. aureus*. These data indicate the lack of cross resistance with penicillin, erythromycin, neamine, neomycin, and celesticetin. The data in table VI in which a streptozotocin-resistant mutant was tested for sensitivity with other antibiotics indicates lack of cross resistance with six other antibiotics.

Physiological Background of Bacterial Resistance to Streptozotocin. Table VII presents data on the rate of disappearance of streptozotocin from cultures of the sensitive and resistant microorganisms. Paper chromatography results indicated only the presence of streptozotocin. Table VII indicates that the concentration of streptozotocin decreased faster in the sensitive *Staph. aureus* parent culture than in the resistant culture. At the end of the 7 hour incubation period, the level of the antibiotic in the tube with the sensitive strain was one half of that with the resistant strain. However, during incubation, the resistant strain was grown to full turbidity and the sensitive strain did not grow as measured by optical density. Some decrease in the concentration of the antibiotic had to be expected, due to its instability at physiological pH and temperature.

In the resistant streak study heavy growth of the streak occurred after 20 hours incubation and faint growth of the seeded sensitive strain was present. This growth of the sensitive strain was obviously due to the inactivation of streptozotocin. There was no stimulation of the sensitive strain in areas of close proximity to the resistant strain. Uniform, heavy growth was found on control plates containing no streptozotocin.

This evidence indicates that the physiological background of the resistance of *Staph. aureus* to streptozotocin is not due to the production of an extracellular enzyme capable of inactivating the antibiotic. Furthermore, the affinity for streptozotocin was decreased in the resistant strain.

SUMMARY

Several aspects of the resistance of two representative species of bacteria, *Staph.*

aureus and *P. vulgaris*, to streptozotocin were investigated and the following conclusions were made.

1. With both microorganisms, resistant mutants were isolated following the first exposure of each organism to the antibiotic when an inoculum of sufficient size was used.

2. The mutants of *Staph. aureus*, resistant to low levels of streptozotocin, as a rule, were resistant to higher levels as well. Streptomycin-like resistance development is indicated. In the case of *P. vulgaris*, however, the gradual (penicillin-like) resistance development seemed to be the prevailing pattern.

3. Resistant mutants did occur in both *Staph. aureus* and *P. vulgaris* at the frequency of one in 10^5 to 10^6 sensitive cells in a 24 hour culture.

4. Streptozotocin was not cross resistant with novobiocin, carbomycin, celesticetin, chloramphenicol, erythromycin, kanamycin, neomycin, penicillin, polymyxin, or tetracycline.

5. It was shown that the development of the resistance was not due to the ability of resistant cells to destroy the drug.

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Rifomycin. I. Isolation and Properties of Rifomycin B and Rifomycin Complex

P. SENSI, A. M. GRECO, AND R. BALLOTTA

Research Laboratories, Lepetit S.p.A., Milan, Italy

Rifomycin has been isolated in our laboratories from the fermentation broths of a strain of *Streptomyces mediterranei* n. sp. and was described in a preliminary report.¹ The morphological characteristics of *S. mediterranei* will be described by Margalith and co-workers in a paper to be published elsewhere.

The fermentation broths of *S. mediterranei* contain several substances displaying antibiotic activity; the previously described rifomycin, which is produced in a larger quantity under certain conditions, has now been named rifomycin B.

Rifomycin B is of interest for its high degree of activity against gram-positive bacteria and *Mycobacterium tuberculosis* and for its very low toxicity; it has promising clinical application. Microbiological and pharmacological properties, as well as preliminary clinical investigations, are presented in other papers.²⁻⁴

The several antibiotic components produced by *S. mediterranei* will be indicated by the letters A, B, C, D, E. They can be separated by paper chromatography using water containing 3 per cent ammonium chloride and 1 per cent ascorbic acid as solvent system. Rifomycin B is easily separated from the other components. This is due to its remarkable acidic nature, its good stability, and the high solubility of its neutral salts; it can be easily crystallized from several solvents. Also, several organic and inorganic salts of rifomycin B crystallize easily.

On the contrary, the other components (A, C, D, E) are neutral or slightly acidic substances; furthermore, they are rather unstable. The separation and purification of these components is very difficult, and we could obtain small quantities of these components only by countercurrent distribution. The crude mixture of these substances shows a high microbiological activity, but its great instability and the variability in the quantitative and qualitative composition of several lots examined make biological evaluation very difficult. This mixture will be indicated as "rifomycin complex" in this and other papers to be published.

In this paper we report the isolation and properties of rifomycin complex and rifomycin B.

EXPERIMENTAL STUDIES

The method of obtaining crystalline rifomycin B and crude rifomycin complex is indicated in figure 1.

PROPERTIES OF RIFOMYCIN COMPLEX

Rifomycin complex has been obtained as an amorphous brown powder. It has very low solubility in neutral or acidic water, but it is soluble at an alkaline pH (9 to 10), giving a dark red solution. Its solubility in water does not exceed 2 per

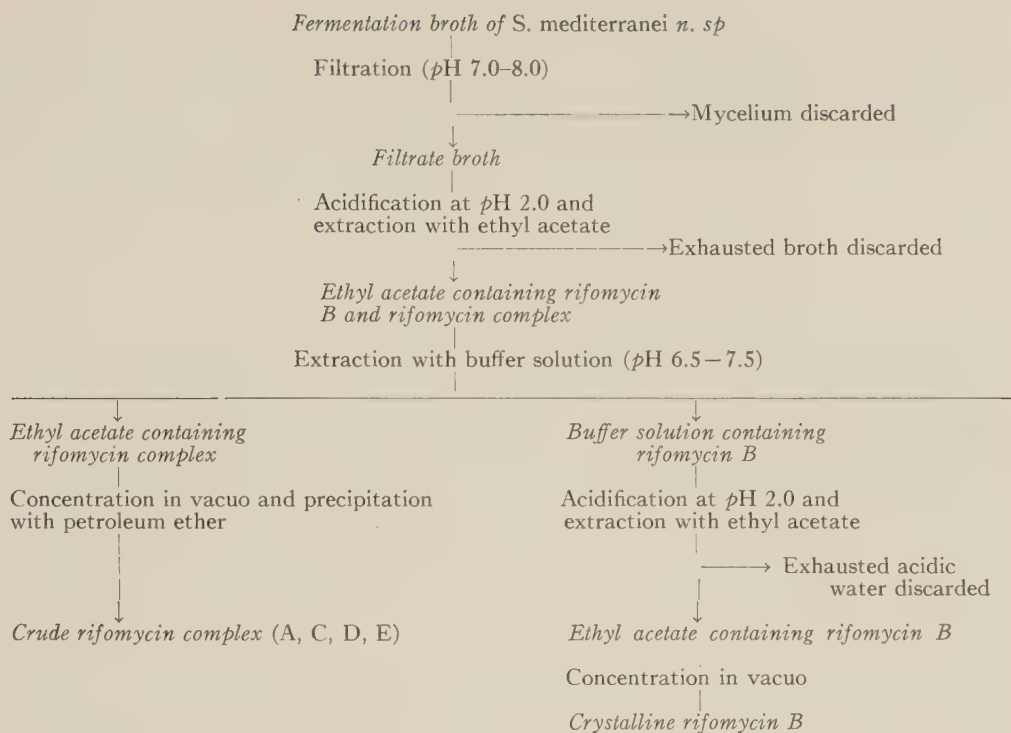


FIG. 1. Method of obtaining crystalline rifomycin B and crude rifomycin complex.

cent in any case. It is soluble in several organic solvents, such as acetone, chloroform, ethyl acetate, methanol, ethanol; it is insoluble in petroleum ether.

The stability of rifomycin complex in acidic, neutral, or alkaline aqueous solutions expressed as residual microbiological activity is shown in table I. At neutral or alkaline pH it is very unstable, but its stability can be greatly improved by the addition of a reducing substance, such as ascorbic acid. However, ascorbic acid increases the instability of rifomycin complex in acidic solutions.

A solution of rifomycin complex at pH 6.0 loses in 24 hours 10 per cent of its microbiological activity at 5 C., 45 per cent at 12 C., 68 per cent at 30 C., and 75 per cent at 37 C. A solution of rifomycin complex at a maximum concentration

TABLE I

*Stability of Rifomycin Complex at 20 C. (Expressed as Per Cent of the
Original Microbiological Activity)*

Hours	Solution of rifomycin complex at 500 γ /ml., pH			Solution of rifomycin complex at 500 γ /ml. + 1% ascorbic acid, pH		
	2.8	6.8	10.0	2.8	6.8	10.0
0	96	76	20.0	96	99	96
6	56	46	12.4	61	98	80
24	50	27	2.0	19	64	70
144	46	6	2.0	4	46	40

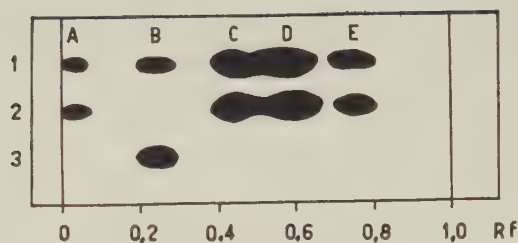


FIG. 2. Paper chromatography using water containing 3 per cent ammonium chloride, 1 per cent ascorbic acid (Whatman no. 1, temperature = 20 C.) (on *Sarcina lutea* plates). (1) total extract from a fermentation broth; (2) rifomycin complex; (3) rifomycin B.

of 2 per cent in a buffer solution pH 9.0 containing ascorbic acid preserves all its microbiological activity when stored at -30°C . for 20 days.

At least four substances with antibiotic activity form the rifomycin complex; they can be separated by paper chromatography or countercurrent distribution. Several solvent systems have been tested to obtain a good separation by paper chromatography. The best and most reliable results were obtained using water containing 3 per cent ammonium chloride and 1 per cent ascorbic acid. Ascorbic acid was added to prevent degradation of some components, particularly C and D. Paper chromatographies of total ethyl acetate extract of a fermentation broth (A, B, C, D, E), of rifomycin complex (A, C, D, E), and of rifomycin B are reported in figure 2.

Countercurrent Distribution of Rifomycin Complex. Using the solvent system consisting of methanol, 0.01 N hydrochloric acid, benzene, petroleum ether (10:5:15:5), not less than 100 transfers are required to separate rifomycin complex into at least four components (fig. 3).

The first 10 tubes contain rifomycin E, and the last 10 tubes rifomycin A. Rifomycin D and C, which are the most important components of rifomycin complex because of their high microbiological activity, are contained respectively between the fortieth to fifty-fifth and sixtieth to seventy-fifth tubes. The various tubes were assayed by microbiological and spectrophotometric methods. Rifomycin A, C, and D show a maximum in the visible region of the spectrum at $460\text{ m}\mu$, while rifomycin E shows a maximum at $400\text{ m}\mu$.

All attempts to obtain these components in a pure crystalline form have so far been unsuccessful.

Only components C and D show an interesting microbiological activity and they have been studied more extensively.

Rifomycin C and D. Rifomycin C and D are brown amorphous substances. They

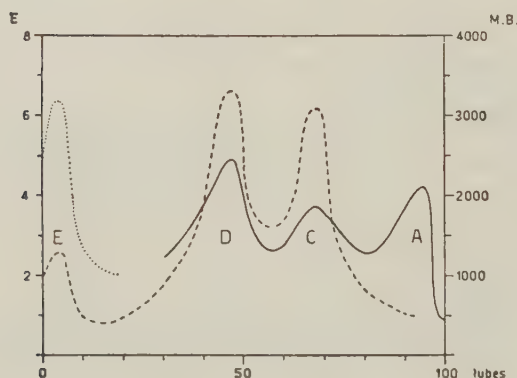
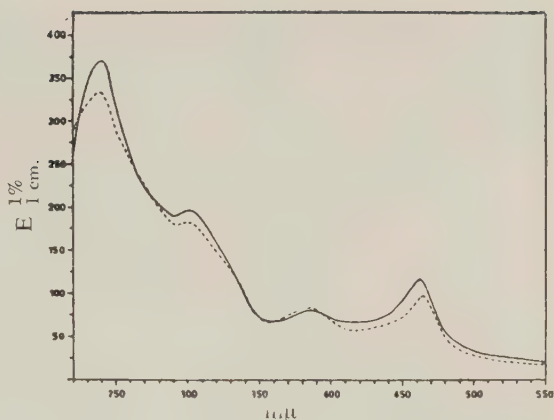


FIG. 3. Countercurrent distribution of rifomycin complex (solvent system: methanol, 0.01 N hydrochloric acid/benzene, petroleum ether [10:5:15:5]). - - - Microbiological assays; spectrophotometric assays at $400\text{ m}\mu$; — spectrophotometric assays at $460\text{ m}\mu$.

FIG. 4. Ultraviolet and visible spectra in methanol of rifomycin C and rifomycin D. — Rifomycin C; ---- rifomycin D.



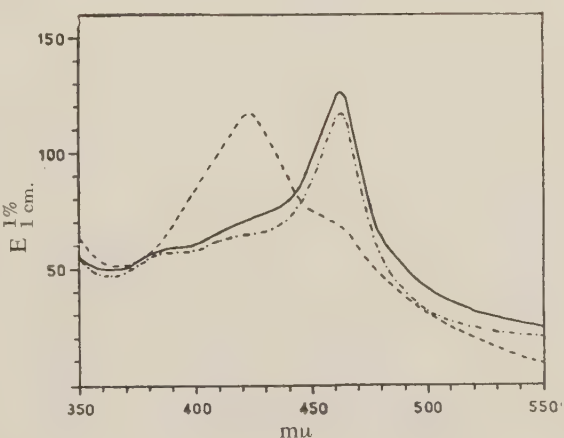
are soluble in methanol, acetone, chloroform, and ethyl acetate, and slightly soluble in neutral or acidic water. They can be dissolved at 2 per cent concentration in buffer solutions at pH 9 to 10 in the presence of ascorbic acid, which preserves them from a rapid inactivation.

The characteristics of rifomycin C and D are similar. Ultraviolet and visible spectra are reported in figure 4, the slight differences being perhaps due to the degree of purity of the two substances. It is worth noting the maximum is in the visible at 460 to 462 $m\mu$. An ethyl acetate solution of rifomycin C or D shows a maximum at the same wave length, but when the solution is shaken with an aqueous solution of a reducing substance, such as ascorbic acid or stannous chloride, the maximum is shifted to 422 $m\mu$ (fig. 5). When the reduced solution is shaken with an aqueous solution of an oxidizing agent, such as potassium ferricyanide, the maximum is again at 460 $m\mu$. These findings indicate that these rifomycins contain an oxidation-reduction system in their molecules. Rifomycin A also shows such a characteristic, which is not shown by rifomycin B and E.

Rifomycin C and D give positive Tollen's, Fehling, and ferric chloride tests, but a negative ninhydrin test. Rifomycin C gives a faintly positive ninhydrin test only after strong acidic hydrolysis.

Elemental analysis is as follows: rifomycin C: C, 61.52; H, 6.73; N, 4.21; rifomycin D: C, 62.17; H, 6.58; N, 3.53. Optical activities cannot be determined because of the strong brown color and the instability of the solutions.

FIG. 5. — A. Absorption spectrum of a rifomycin C solution in ethyl acetate. ---- B. Solution A shaken with 1 per cent stannous chloride solution. - - - C. Solution B shaken with 0.1 per cent potassium ferricyanide solution. (The same behavior is shown by rifomycin D and rifomycin A.)



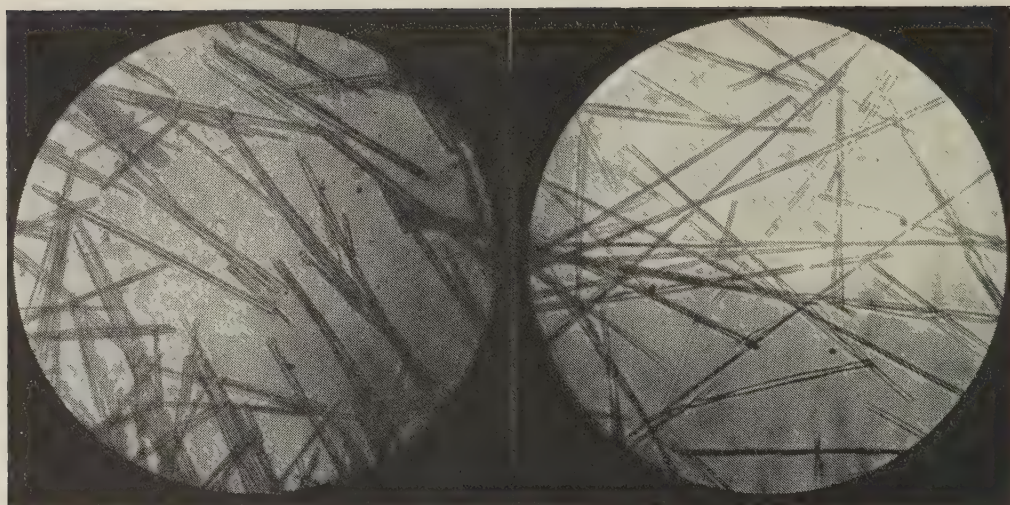


FIG. 6. (Left) Crystals of rifomycin B.

FIG. 7. (Right) Crystals of rifomycin B from a slowly cooled benzene solution.

PROPERTIES OF RIFOMYCIN B

Crystalline Form. Rifomycin B generally crystallizes as brilliant yellow prismatic needles (fig. 6). If a warm saturated solution is slowly cooled, very long, thin needles of rifomycin B are formed (fig. 7).

Melting Point. Rifomycin B has no definite melting point. It decomposes at 160 to 164 C. and does not melt until 300 C.

Solubility. Rifomycin B is scarcely soluble in most usual solvents: water, 0.027 per cent; methanol, 2.62 per cent; ethanol, 0.44 per cent; acetone, 0.31 per cent; chloroform, 0.34 per cent; benzene, 0.018 per cent; ethyl acetate, 0.19 per cent; carbon tetrachloride, 0.0011 per cent; ethyl ether, 0.005 per cent; petroleum ether, <0.005 per cent.

Nature. Rifomycin B is a dibasic acid; electrometric titration in methanol-water shows two acidic groups, $pH_1^{1/2} = 2.8$ (equivalent weight 780), $pH_2^{1/2} = 6.7$ (equivalent weight 765).

Analysis. Analysis is as follows: C, 61.75; H, 6.72; N, 1.88; O, 29.22; OCH_3 ,

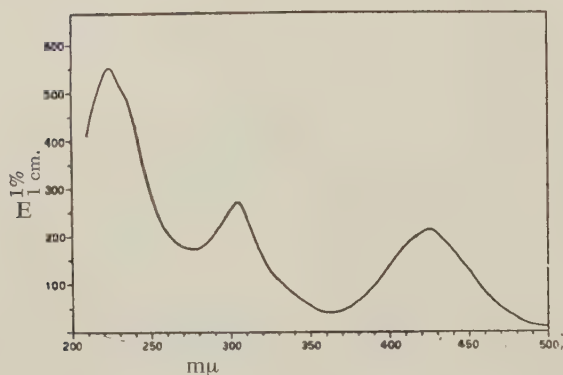


FIG. 8. Ultraviolet and visible spectrum of rifomycin B in phosphate buffer solution pH 7.3.

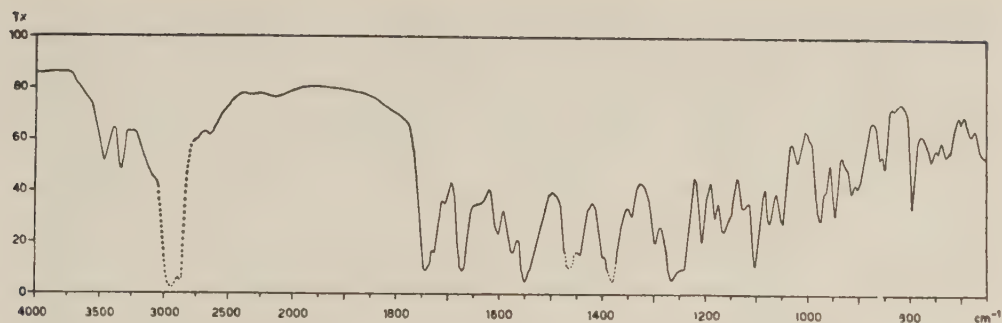


FIG. 9. Infrared spectrum of rifomycin B (in Nujol).

4.03; COCH_3 , 5.51 (calculated for $\text{C}_{39}\text{H}_{51}\text{NO}_{14}$ [proposed] : C, 61.8; H, 6.78; N, 1.85; O, 29.56; 1 OCH_3 , 4.09; 1 COCH_3 , 5.68).

Molecular Formula and Weight. From the elemental analysis and potentiometric titrations, the most probable formula is $\text{C}_{39}\text{H}_{51}\text{NO}_{14}$, molecular weight 757.81. A determination of molecular weight in cyclohexanol (Rast's method) gave an approximate value of 750.

Optical Activity. Optical activity is $[\alpha]_{589}^{20} = -11^\circ$ ($c = 1$ per cent in methanol), determined in a photoelectric polarimeter.

Ultraviolet and Visible Spectrum. In a buffer phosphate solution with pH 7.3, rifomycin B shows an absorption at 223 $\text{m}\mu$ ($E_{1\text{cm.}}^{1\%} = 555$); 304 $\text{m}\mu$ ($E_{1\text{cm.}}^{1\%} = 275$); 425 $\text{m}\mu$ ($E_{1\text{cm.}}^{1\%} = 220$) (fig. 8).

Infrared Spectrum. The crystals obtained from ethyl acetate show the following absorption bands in Nujol suspension: 3465, 3330, 1742, 1730 (shoulder), 1705 (shoulder), 1673, 1602, 1575, 1550, 1445, 1342, 1300, 1267, 1240 (shoulder), 1205, 1180, 1165, 1102, 1073, 1048, 1020, 975, 948, 915, 905, 850, 795, 760, 730, and 680 cm^{-1} (fig. 9). The infrared spectrum of crystals obtained from methanol or benzene is slightly different.

Stability. Crystalline rifomycin B is very stable and may be kept at room temperature for several months without significant alteration.

The stability of the solutions of its monosodium (pH 4.0) and disodium (pH 7.2) salt at 4 and 37 C. is reported in table II. Rifomycin B is very stable in neutral solution; as the monosodium salt (pH 4.0) its stability is not so good, particularly

TABLE II

Stability of Rifomycin B (Concentration 1000 $\gamma/\text{ml.}$)
(Expressed as Recovery Percentage of the Original Rifomycin B)

Hours	Temperature = 4 C., pH		Temperature = 37 C., pH	
	4.0	7.2	4.0	7.2
0	100	99	99	100
16	98	100	76	98
24	97	100	68	97
40	94	98	60	92
64	94	96	43	88
88	90	99	33	85
160	82	98	12	79

at 37 C. During the stability tests, assays of rifomycin B were performed, extracting and weighing the crystalline rifomycin B obtained. We adopted this technique because during these stability tests, the microbiological assays showed an increase of the microbiological activity of the solution, particularly at pH 4.0. This behavior, quite unusual and surprising, is certainly due to the conversion of rifomycin B into another substance with higher antibiotic activity. We will discuss this substance in another paper to be published.

Paper Chromatography. The R_f values in several solvent systems are: water containing 3 per cent ammonium chloride, 1 per cent ascorbic acid: 0.25; butanol saturated with water: 0.87; water saturated with butanol: 0.87; butanol, acetic acid, water (4:1:5): 0.95; butanol, acetic acid, ethanol, water (25:3:25:47): 0.85; acetone, water (1:1): 0.88; chloroform, cyclohexane, water (8:1:2): 0.92.

Chemical Properties. The dibasic acid character of rifomycin B is related to the presence of a carboxylic and an enolic group in its molecule. Rifomycin B reduces neutral and acidic permanganate solutions, gives positive ferric chloride, Tollens and Fehling tests, absorbs bromine in carbon tetrachloride solutions, and gives a yellow precipitate when treated with iodine in alkaline solution. Ninhydrin test is negative, but faintly positive after strong acidic hydrolysis. Rifomycin B contains a methoxyl and an acetyl group in its molecule. It absorbs about 4 moles of hydrogen in the presence of Adams' catalyst. With dinitrophenylhydrazine it gives a bis-dinitrophenylhydrazone, and when treated with acetic anhydride in a pyridine solution, it gives a triacetyl derivative. With diazomethane it gives a trimethyl derivative, which can be acetylated to monoacetyl-trimethyl derivative.

Triacetyl rifomycin B is obtained as pale yellow crystals. The melting point is 155 to 158 C., $[\alpha]_{589}^{20} = +38.5$ degrees ($c = 1$ per cent methanol). It is a monobasic acid, $pK = 4.85$. It is soluble in usual organic solvents with greenish fluorescence. It is insoluble in water, soluble in alkaline solutions. Analysis shows: C, 61.80; H, 6.64; N, 1.77; O, 29.26; COCH_3 , 19.34; OCH_3 , 3.48 (calculated for $\text{C}_{15}\text{H}_{57}\text{NO}_{17}$ [proposed] = C, 61.14; H, 6.50; N, 1.58; O, 30.77; 4 COCH_3 , 19.48; 1 OCH_3 , 3.51).

Trimethyl rifomycin B is obtained as pale yellow crystals. The melting point is 126 to 131 C., $[\alpha]_{589}^{20} = +79.4$ degrees ($c = 1$ per cent in methanol). It is a neutral substance, insoluble in alkaline or acidic water, soluble in usual organic solvents. Analysis shows: C, 63.24; H, 7.52; N, 1.98; COCH_3 , 5.20; OCH_3 , 15.90 (calculated for $\text{C}_{42}\text{H}_{57}\text{NO}_{14}$ [proposed] = C, 63.06; H, 7.18; N, 1.75; 1 COCH_3 , 5.38; 4 OCH_3 , 15.52).

Trimethyl-monoacetyl rifomycin B is obtained as pale yellow crystals. The melting point is 125 to 130 C., $[\alpha]_{589}^{20} = +68.6$ degrees ($c = 0.7$ per cent in methanol). It is a neutral substance, insoluble in water, soluble in usual organic solvents. Analysis shows: C, 62.38; H, 7.38; N, 2.0; COCH_3 , 9.5; OCH_3 , 14.40 (calculated for $\text{C}_{44}\text{H}_{59}\text{NO}_{15}$ [proposed] = C, 62.76; H, 7.06; N, 1.66; 2 COCH_3 , 10.22; 4 OCH_3 , 14.74).

Rifomycin B-bisdinitrophenylhydrazone is obtained as red crystals. The melting point is 167 C. (decomposition). Analysis shows: C, 55.29; H, 5.52; N, 10.97 (calculated for $\text{C}_{51}\text{H}_{59}\text{N}_9\text{O}_{20}$ [proposed] = C, 54.78; H, 5.32; N, 11.27).

Neutral and Monobasic Salts. As a dibasic acid, rifomycin B forms neutral and monobasic salts with organic and inorganic bases.

The monosodium salt is prepared by adding to a methanolic suspension of rifomycin B the equimolecular quantity of sodium methoxide. Rifomycin B dissolves completely and then precipitates as yellow-orange crystals. The solubility of the monosodium rifomycin B in water is about 0.7 per cent (pH 4.1). Analysis shows: Na, 2.81 per cent (calculated 2.95); rifomycin B, 95.3 per cent (calculated 97.05).

Calcium, magnesium, and ammonium monobasic salts of rifomycin B are obtained as yellow-orange crystals from the aqueous solution of the monosodium salt, adding an excess of calcium, magnesium, or ammonium chloride.

The solubility of these salts in water is very low (<0.1 per cent), pH 4.0 to 4.1. The calcium monobasic salt is Ca, 2.55 per cent (calculated 2.58); rifomycin B, 95.0 per cent (calculated 97.42). The magnesium monobasic salt is Mg, 1.5 per cent (calculated 1.58); rifomycin B, 95.0 per cent (calculated 98.42). The ammonium monobasic salt is N, 3.6 per cent (calculated 3.62); rifomycin B, 93.03 per cent (calculated 98.78).

The sodium, magnesium, and ammonium neutral salts are very soluble, particularly the disodium salt (pH 7.0). It is difficult to obtain these salts in a crystalline form. The calcium salt has about 2 per cent solubility in water (pH 6.5) and crystallizes easily. Analysis shows Ca, 5.30 per cent (calculated 5.03); rifomycin B, 93.2 per cent (calculated 94.97). The preparation of slightly soluble organic salts is easily performed by adding the hydrochloride of the suitable organic base to a neutral solution of sodium rifomycin B. Some crystalline neutral salts of rifomycin B with organic bases were prepared.

The quinine salt is C, 65.1; H, 6.92; N, 3.90 (calculated for $C_{20}H_{24}N_2O_2 \cdot C_{39}H_{51}NO_{14}$; C, 65.47; H, 6.98; N, 3.88); rifomycin B, 68.3 per cent (calculated 70.02 per cent). Solubility is 0.13 per cent.

The dibenzylamine salt is C, 69.2; H, 6.86; N, 3.71 (calculated for $(C_{14}H_{15}N)_2 \cdot C_{39}H_{51}NO_{14}$; C, 69.83; H, 7.08; N, 3.65); rifomycin B, 64.5 per cent (calculated 65.76). Solubility is 0.32 per cent.

The dibenzylethylenediamine salt is C, 66.3; H, 7.2; N, 4.05 (calculated for $C_{16}H_{20}N_2 \cdot C_{39}H_{51}NO_{14}$; C, 66.17; H, 7.17; N, 4.21); rifomycin B, 79.8 per cent (calculated 75.92). Solubility is 0.12 per cent.

The 2-methyl-6-aminoheptane salt is C, 64.8; H, 8.9; N, 4.2 (calculated for $(C_8H_{19}N)_2 \cdot C_{39}H_{51}NO_{14}$; C, 65.00; H, 8.83; N, 4.13); rifomycin B, 74.6 per cent (calculated 74.56). Solubility is 0.17 per cent.

SUMMARY

The previously described rifomycin¹ is one of a group of antibiotics produced by *S. mediterranei* n. sp. and has now been named rifomycin B.

Rifomycin B is easily separated from the other components because of its remarkable acidic character and good stability.

Rifomycin complex, which is produced together with rifomycin B in the fermentation process, is a mixture of several substances, most of them showing high microbiological activity. The isolation and purification of the components of rifomycin complex is difficult because of their instability. Paper chromatography, countercurrent distribution, and other properties of rifomycin complex are reported. The most active components of rifomycin complex (rifomycin C and D) are characterized by the presence of an oxidation-reduction system in the molecules.

Rifomycin B is obtained in a pure crystalline form.

The chemical and physical properties of rifomycin B are described in detail. It is a dibasic acid and forms neutral or monobasic salts. The preparation and properties of salts of rifomycin B with inorganic and organic bases, as well as of some derivatives of rifomycin B, are reported.

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Rifomycin. II. Antibacterial Activity of Rifomycin B

M. T. TIMBAL

Research Laboratories, Lepetit S.p.A., Milan, Italy

In previous papers^{1,2} the isolation and the physical and chemical properties of a new antibiotic, rifomycin, have been described. As has been stated in these papers, five microbiologically active components have been identified in the fermentation broths of *Streptomyces mediterranei*. The data concerning the so-called rifomycin complex will be reported elsewhere; in this work the fraction rifomycin B in pure crystalline form was used.

IN VITRO PROPERTIES

Antibacterial Spectrum. The determination of the minimal inhibitory concentration was carried out by dilution method in nutrient broth. Bacterial inocula were prepared by diluting an 18 hour broth culture 1000-fold for most of the test organisms and 100-fold when slow-growing organisms were used; 0.1 per cent of this diluted suspension was then inoculated in the nutrient broth. For mycobacteria and fungi, inoculum was prepared by washing a fresh slant and adding 0.5 per cent of homogeneous suspension. Media used for the determination are indicated in table I. Bacteria and mycobacteria were incubated at 37 C. and observation was made after 18 hours, except for *Mycobacterium tuberculosis*; for this organism the incubation time was seven days. Fungi were incubated at 28 C. for 48 hours.

In every experiment a fresh solution of rifomycin B was prepared in phosphate buffer pH 7.2. It will be seen (table I) that rifomycin B is very active against gram-positive cocci, *Myco. tuberculosis* and *Mycobacterium phlei*. It shows moderate activity against bacilli and the other acid-fast organisms. Against fungi and gram-negative organisms the activity is very low. However, even a low activity against gram-negative bacteria could be interesting for practical purposes, since concentrations as high as 5000 to 10,000 µg./ml. are found in the bile (of rats, dogs, and man) within the first four to five hours after administration.^{3,4}

Cross-Resistance Studies. The activity of rifomycin B against antibiotic-resistant strains was determined by the tube dilution technique with Penassay broth. *Micrococcus pyogenes* var. *aureus* ATCC 6538 was made resistant to various antibiotics in our laboratory. Results of these experiments are reported in table II.

It will be seen that rifomycin B shows the same activity against the resistant strains and the sensitive strains. The amount of 2 µg./ml. necessary to inhibit the growth of the oleandomycin-resistant strain 6538/0 cannot be considered as evidence of very appreciable cross resistance between rifomycin B and oleandomycin.

Influence of Serum. The influence of various amounts of bovine serum, added to standard media, on the minimal inhibitory concentration of rifomycin B for several organisms was also studied. The results are indicated in table III. Within the range of variability of a dilution test, one may conclude that serum has no effect on rifomycin B.

TABLE I

Minimal Inhibitory Concentration

Organism	Medium (Difco)	Minimal inhibitory concentration, $\mu\text{g./ml.}$
<i>Micrococcus pyogenes</i> var. <i>aureus</i> ATCC 6538	Penassay broth	0.025
<i>Micrococcus pyogenes</i> var. <i>aureus</i> ATCC 13301	Penassay broth	0.1
<i>Micrococcus pyogenes</i> var. <i>aureus</i> ATCC 9144	Penassay broth	0.025
<i>Micrococcus pyogenes</i> var. <i>albus</i> ATCC 12228	Penassay broth	0.025
<i>Micrococcus pyogenes</i> var. <i>aureus</i> gray M 551	Penassay broth	0.1
<i>Streptococcus faecalis</i> ATCC 10541	Penassay broth	0.5
<i>Streptococcus hemolyticus</i> C 203	Brain-heart infusion	0.025
<i>Streptococcus mastitidis</i> ATCC 7077	Brain-heart infusion	0.5
<i>Streptococcus bovis</i> ATCC 9809	Brain-heart infusion	0.25
<i>Neisseria catarrhalis</i> ATCC 8176	Tryptose phosphate broth	0.5
<i>Neisseria gonorrhoeae</i> ATCC 9826	Tryptose phosphate broth	2
<i>Diplococcus pneumoniae</i> XXVII L	Brain-heart infusion	0.05
	2% serum	
<i>Sarcina lutea</i> ATCC 9341	Penassay broth	0.1
<i>Sarcina subflava</i> ATCC 7468	Penassay broth	0.5
<i>Micrococcus flavus</i> ATCC 10240	Penassay broth	0.25
<i>Bacillus subtilis</i> ATCC 6633	Penassay broth	2.5
<i>Bacillus cereus</i> ATCC 10876	Penassay broth	5
<i>Bacillus anthracis</i> M 401	Penassay broth	1
<i>Clostridium perfringens</i> ATCC 3226	AC broth	0.5
<i>Klebsiella pneumoniae</i> ATCC 10031	Penassay broth	1000
<i>Klebsiella pneumoniae capsulata</i> M 117	Penassay broth	2500
<i>Escherichia coli</i> McLeod ATCC 10536	Penassay broth	1000
<i>Pseudomonas aeruginosa</i> Gottlieb	Penassay broth	2000
<i>Pseudomonas fluorescens</i> ATCC 11251	Penassay broth	500
<i>Proteus vulgaris</i> X 19 H ATCC 881	Penassay broth	1000
<i>Proteus morganii</i> ATCC 9237	Penassay broth	2500
<i>Proteus rettgeri</i> ATCC 9919	Penassay broth	1000
<i>Shigella sonnei</i> ATCC 9290	Penassay broth	5000
<i>Shigella dysenteriae</i> ATCC 9583	Penassay broth	500
<i>Salmonella typhi</i> M 507	Penassay broth	2500
<i>Salmonella paratyphi</i> ATCC 9150	Penassay broth	2500
<i>Salmonella shottmuelleri</i> ATCC 9149	Penassay broth	5000
<i>Brucella abortus</i> M 311	Brain-heart infusion	150
<i>Brucella melitensis</i> ATCC 4309	Brain-heart infusion	75
<i>Pasteurella pestis</i> ATCC 87 NIH	Brain-heart infusion	100
<i>Mycobacterium ranae</i> M 215	TB broth base	50
<i>Mycobacterium phlei</i> ATCC 10142	TB broth base	0.05
<i>Mycobacterium minetti</i> M 421	TB broth base	100
<i>Mycobacterium species</i> 607	TB broth base	100
<i>Mycobacterium tuberculosis</i> var. <i>hominis</i> H ₃₇ Rv ATCC 9360	Proskauer medium	0.05
<i>Nocardia asteroides</i> CBS	Sabouraud broth	350
<i>Saccharomyces cerevisiae</i> ATCC 9763	Sabouraud broth	>2500
<i>Candida albicans</i> ATCC 10231	Sabouraud broth	>2500
<i>Kloeckera brevis</i> ATCC 9774	Sabouraud broth	350
<i>Cryptococcus neoformans</i> SKF 1110	Sabouraud broth	>2500
<i>Blastomyces dermatitidis</i> SKF 450	Sabouraud broth	625
<i>Trichosporon cutaneum</i> ATCC 4155	Sabouraud broth	>2500
<i>Histoplasma capsulatum</i> SKF 2180	Sabouraud broth	>2500
<i>Aspergillus niger</i> M 504	Sabouraud broth	>2500
<i>Trichophyton mentagrophytes</i> SKF 17410	Sabouraud broth	>2500
<i>Trichophyton mentagrophytes</i> SKF 8410	Sabouraud broth	>2500
<i>Trichophyton schoenleinii</i> ATCC 4822	Sabouraud broth	>2500

TABLE II

*Rifomycin B Sensitivity of Antibiotic-Resistant Strains of M. pyogenes var. aureus:
Resistance Pattern*

Strain	Resistant to units or $\mu\text{g.}/\text{ml.}$ of following antibiotics		Rifomycin B, minimal inhibitory concentration, $\mu\text{g.}/\text{ml.}$
6538/P	Penicillin	100 units/ml.	0.05
6538/E	Erythromycin	100 $\mu\text{g.}/\text{ml.}$	0.1
6538/N	Novobiocin	100 $\mu\text{g.}/\text{ml.}$	0.05
6538/O	Oleandomycin	100 $\mu\text{g.}/\text{ml.}$	2
6538/S	Streptomycin	1000 $\mu\text{g.}/\text{ml.}$	0.1
6538/C	Chloramphenicol	100 $\mu\text{g.}/\text{ml.}$	0.05
6538/T	Tetracycline	100 $\mu\text{g.}/\text{ml.}$	0.1
6538	Sensitive control		0.05

Influence of Inoculum Size. Dilution series of rifomycin B were prepared and inoculated with 3×10^1 , 3×10^2 , 3×10^3 , and 3×10^4 cells/ml. of an antibiotic-sensitive *M. pyogenes var. aureus*. The tubes were incubated at 37 C. and examined for visual growth at 24 hour intervals up to 72 hours. The results indicate (table IV) that no or very little inoculum effect exists within the studied range of inoculum size.

With greater inocula we observed the possible occurrence of "skips" in tubes containing concentrations of rifomycin B in excess of the minimal inhibitory concentration. These "skips" could be due to resistant mutants.

We have therefore tried to determine the proportion of spontaneous mutant resistant cells in strains of *M. pyogenes var. aureus* and *Streptococcus hemolyticus* by counting the number of colonies grown in 24 hours at 37 C. on agar containing 10 and 100 $\mu\text{g.}/\text{ml.}$ The ratio of bacterial cells, present in our standard strains, capable of growing at 10 and 100 $\mu\text{g.}/\text{ml.}$ is of the order respectively of 10^{-5} and 10^{-6} for *M. pyogenes var. aureus* and of $<5 \times 10^{-9}$ for both concentrations for *Str. hemolyticus*.

Studies concerning the rate of spontaneous mutation of sensitive cells, the development of in vitro resistance by transfers, and the analysis of the bactericidal effect of our antibiotic versus the bacteriostatic effect are made particularly difficult by the phenomenon we call "in vitro activation." These studies will therefore be discussed in conjunction with a detailed analysis of this phenomenon.

Activation of the Microbiological Potency of Rifomycin B. At the beginning of our studies on rifomycin B it appeared that this antibiotic showed a paradoxical

TABLE III

*Effect of Bovine Serum on the Activity of Rifomycin B
(Minimal Inhibitory Concentration, $\mu\text{g.}/\text{ml.}$)*

Microorganism	Per cent bovine serum added			
	0	5	15	45
<i>M. pyogenes var. aureus</i> ATCC 6538	0.025	0.025	0.1	0.25
<i>M. pyogenes var. albus</i> ATCC 12228	0.025	0.025	0.05	0.025
<i>Str. faecalis</i> ATCC 10541	0.5	0.5	1	0.5
<i>Str. hemolyticus</i> C 203	0.05	0.025	0.025	0.05
<i>S. lutea</i> ATCC 9341	0.05	0.1	0.25	0.1

TABLE IV

The Effect of Inoculum Size on the Activity of Rifomycin B Against M. pyogenes var. aureus

Cell inoculum	Minimal inhibitory concentration, $\mu\text{g.}/\text{ml.}$		
	24 hours	48 hours	72 hours
3×10^1	0.025	0.05	0.05
3×10^2	0.025	0.05	0.05
3×10^3	0.025	0.1	0.1
3×10^4	0.05	0.1	0.1

"activation" of the microbiological potency in solution. A typical experiment is shown in table V. The solution of rifomycin B in phosphate buffer pH 7.0 was prepared at a concentration of 1000 $\mu\text{g.}/\text{ml.}$ and kept at 37 C. for three days. At 12 hour intervals the solution was tested against a freshly prepared standard of rifomycin B, using the paper disc method. The most obvious hypothesis on the mechanism of "activation" is that there is a production of a derivative either having a higher antibacterial activity or showing a higher diffusion rate in agar. Paper chromatography of the activated solution (*n*-amyl alcohol:*n*-butyl alcohol [9:1] saturated with 1 per cent aqueous ascorbic acid) showed very clearly that a new compound, different from rifomycin B, was produced during the activation process (fig. 1).

Good indirect evidence is now available that the derivative should be many times more active than the original antibiotic. It has already been reported² that, under the same conditions of our experiment, after 72 hours, 85 to 88 per cent of the original rifomycin B can be recovered as unaltered compound. This indicates that the new derivative is, under our experimental conditions, at least 20 to 25 times more active than rifomycin B.

IN VIVO ACTIVITY

Albino mice of CF-1 strain weighing 18 to 20 Gm. were used for the protection test against *Str. hemolyticus* C 203, *Diplococcus pneumoniae* XXVII L, and coagulase-positive *Staphylococcus pyogenes* var. *aureus* gray M 551.

The mice were infected intraperitoneally with 5 to 30 LD₁₀₀ doses of the organism. Mucin was employed as an adjuvant with *Staphylococcus*. Fresh solutions of rifomycin B in phosphate buffer pH 7.2 were administered subcutaneously in the

TABLE V

Activation of Rifomycin B

Hours at 37 C.	Potency, $\mu\text{g.}/\text{ml.}$
0	1000
12	1850
24	2600
48	3320
72	3960

FIG. 1. Paper chromatography of a fresh solution (1) and a 72 hour solution (2) of rifomycin B.



amount of 0.2 ml./injection. Preliminary experiments showed that the antibiotic would give better results when administered in four doses daily, every six hours, rather than in two daily doses.

Independent tests were carried out administering the antibiotic either immediately after the infection or six hours later. The data are presented in table VI.

All the mice that survived at the end of the experiment were sacrificed and abscess formation and other gross pathology, if visible, were observed. The mice

TABLE VI
The Efficacy of Rifomycin B in the Treatment of Infected Mice

Organism	Beginning of treatment after infection, hours	Daily dose, mg./Kg.	Survivors		ED ₅₀ , mg./Kg. (confidence limits)
			No.	%	
<i>Str. hemolyticus</i> C 203	0	150	15/15	100	96 (109-84)
	0	125	13/15	87	
	0	100	9/15	60	
	0	75	1/15	7	
	0	50	0/15	0	
<i>Str. hemolyticus</i> C 203	6	300	19/20	95	170 (194-149)
	6	250	18/20	90	
	6	200	14/20	70	
	6	150	6/20	30	
	6	100	1/20	5	
<i>D. pneumoniae</i> XXVII L	0	200	20/20	100	110 (132-92)
	0	150	17/20	85	
	0	125	13/20	65	
	0	100	8/20	40	
	0	75	2/20	10	
<i>D. pneumoniae</i> XXVII L	6	300	20/20	100	190 (211-172)
	6	250	18/20	90	
	6	200	12/20	60	
	6	150	2/20	10	
<i>Staph. pyogenes</i> var. <i>aureus</i> gray M 551	0	400	13/14	93	305 (332-279)
	0	350	9/14	64	
	0	300	6/14	43	
	0	250	4/14	14	
	0	200	1/14	0.7	

infected with *Str. hemolyticus* but still surviving did not show any pathological alteration, and no pathogenic organism was isolated from blood or organs.

The animals surviving after infection from *D. pneumoniae* showed paralysis and other symptoms related to encephalitis. These results are in agreement with the results obtained on pharmacological investigation,³ showing that the antibiotic cannot be detected in the cerebrospinal fluid.

In staphylococcal infections some abscesses were also found in the liver and in the kidney after 12 days of treatment in 20 to 60 per cent of the surviving animals.

Even if the ED₅₀ is not so favorable as in the case of other well-known antibiotics, the results reported here can be considered quite good in view of the fact that rifomycin B has a very low toxicity.

SUMMARY

Some of the microbiological properties of the new antibiotic rifomycin B are described. It is active in vitro against a variety of gram-positive cocci at concentrations as low as 0.01 to 0.5 µg./ml. It does not show activity against gram-negative organisms and fungi. Mycobacteria are inhibited, particularly *Myco. tuberculosis* var. *hominis*.

Bovine serum added to a standard broth does not influence the minimal inhibitory concentration and rifomycin B shows no cross resistance to other antibiotic-resistant strains. The influence of the inoculum size and development of resistant cells are discussed.

Water solution of rifomycin B undergoes an "activation" of the microbiological potency due to the transformation of a small portion of the antibiotic into a new, much more active derivative.

Experimental infections with different types of gram-positive cocci are completely controlled by subcutaneous administration of rifomycin B.

According to the procedure used for therapeutic treatment, the ED₅₀ values range between 96 and 170 mg./Kg. for *Str. hemolyticus*, between 110 and 190 mg./Kg. for *D. pneumoniae*. For *Staph. aureus* gray the ED₅₀ value is 305 mg./Kg.

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Rifomycin. III. Pharmacology of Rifomycin B

GIULIO MAFFII AND MARIA T. TIMBAL

Research Laboratories, Lepetit S.p.A., Milan, Italy

Rifomycin B is a new antibiotic substance obtained from *Streptomyces mediterranei* and well characterized by physicochemical point of view.^{1,2} Its antibacterial potency and effectiveness against numerous gram-positive organisms appeared very high in degree from microbiological studies³ and comparable to that of the most actively known antibiotics. This activity coupled with a low toxicity indicates that it is a potentially valuable chemotherapeutic agent worthy of adequate pharmacological analysis.

Herein the results of a preliminary study of the effects of rifomycin B on laboratory animals are reported.

MATERIALS AND METHODS

Mice CF-1, rats CF Wistar, mongrel dogs, and cats were used in these experiments. For LD₅₀ values in acute toxicity studies, the method of Litchfield and Wilcoxon⁴ was followed. Usually four or more doses were administered to groups of 10 or 20 animals each. Intravenous injections in mice and rats were performed at a rate of 0.1 ml./sec. Deaths were referred to the antibiotic if occurring within five days following the administration. Current laboratory methods were employed for blood counts and blood sugar levels, serum protein electrophoresis, coagulation time, and urine analysis, in subacute and chronic toxicity tests.

All the restricted animals in excretion and in subacute toxicity studies received food and water ad libitum.

Blood pressure in anaesthetized dogs was registered by means of a pressure transducer connected with a Sanborn recorder apparatus. Respiration was recorded through a pneumograph fixed to thoracic walls and connected with a pressure transducer. The transducer itself was then connected with the Sanborn recording apparatus.

For bile excretion studies, rats were anaesthetized with ether and the biliary duct surgically cannulated. In dogs under pentobarbital sodium (40 mg./Kg. intravenously) anesthesia, bile was collected through a polystan tube inserted in the hepatic duct (gall bladder and choledochus being excluded). A catheter was introduced, through the urethra, into the urinary bladder for collecting urine.

For microbiological assays of blood serum samples, collected during this investigation, the filter paper discs method was used. The test organism was *Micrococcus pyogenes* var. *aureus* ATCC 6538. Culture media was a nutrient agar to which was added 10 per cent of M 1 phosphate buffer pH 4.0. The corrected pH of the medium was 5.0. Solutions of the working standards were prepared by diluting in bovine serum a fresh stock solution of rifomycin B. The unusual pH of the medium for microbiological assays was found to be the most suitable for enhancing the sensitivity of the test and therefore for a quantitative evaluation of antibiotic concentrations as low as 0.1–0.05 µg./ml. In addition to this the assay

TABLE I
Acute Toxicity of Rifomycin B

Species	Route	LD ₅₀ in mg./Kg. (19/20 confidence limits)
Mouse	Intravenous	2040 (1924–2162)
	Intraperitoneal	>3000
	Subcutaneous	>3000
	Oral	>3000
Rat	Intravenous	1680 (1527–1848)
	Intraperitoneal	>3000
	Subcutaneous	>3000
	Oral	>3000
Dog	Intravenous	About 1200
Guinea pig	Intraperitoneal	About 3000

method used was proved to be the best in order to avoid the cause of error due to the rifomycin B activation.³ Details on this complicated problem of the microbiological activity of rifomycin will be discussed in further reports.

The same method was used for the assay of the other body fluids, such as bile, urine, and liquor.

We are not completely sure that the data here presented concerning the levels of antibiotic activity in biological media are to be considered exclusively due to rifomycin B. Studies on the stability of rifomycin B,² however, suggest that only a small portion of the antibiotic has undergone spontaneous changes during the period of the biological tests.

In all the experiments crystalline sodium salt of rifomycin B was used. To prepare the proper solutions of the antibiotic *N*/1 sodium hydroxide was added until a final *pH* of 7.5 to 7.7 was reached. The antibiotic was always administered not more than 15 minutes after the solutions were prepared.

ACUTE TOXICITY

The intravenous LD₅₀ of rifomycin B has been determined in mice, rats, and dogs (table I). In mice and rats most deaths occurred within four hours and were preceded by convulsions and gasping respiratory movements. The surviving animals were kept under observation for one week. In dogs dyspneic respiration, agitation, muscular twitches, defecation, and emesis were observed after injections of toxic doses of rifomycin B. Death or complete recovery occurred 15 to 60 minutes after the injection. Doses up to 3 Gm./Kg. were well tolerated by mice and rats, either by subcutaneous or intraperitoneal administration. However, subcutaneous, intramuscular, and intraperitoneal injection of these high doses produces local irritation and phlogistic damage of tissues.

SUBACUTE TOXICITY

Fifteen young male rats were given 2000 mg./Kg. of rifomycin B once a day by intraperitoneal injection for 20 days without evidence of toxicity. A control group received corresponding volumes of distilled water. The average body weight, as well as the weight of dissected organs (liver, kidneys, thymus, adrenals, and testes) and complete blood counts, as determined at the conclusion of the study, indicated that there were no significant alterations in the animals treated. Two additional groups

of 10 rats each received single daily subcutaneous doses of 500 and 2000 mg./Kg. for 20 days without evidence of toxicity. However, signs of local irritation were observed in subcutaneous tissues of the sites of injection (edema, hemorrhage, induration, and coloration of tissues).

Two dogs were given intramuscular daily doses of 250 mg./Kg. for a period of 44 days. Except for signs of local irritation at the site of injection, the dogs remained symptom-free. Appetites were normal. Urine analysis, blood nitrogen levels, the Takata-Ara test for liver function, serum protein electrophoretic patterns, blood sugar levels, the Quick-time, the bleeding time, and complete blood counts during and at the conclusion of the experiments did not differ significantly from predosing observations. Gross examination of the autopsied animals at the end of the study showed signs of severe irritation in the sites of injection. Edema, infiltration, hemorrhage, and, in one animal, a sterile abscess with unadsorbed product were found. Gross and microscopic examination of the viscera of these animals showed a low degree degeneration in liver and kidneys. However, the local irritation as well as the evidence of unadsorbed fraction of the injected antibiotic lets the validity of toxicity data thus obtained open to further question.

Two additional dogs were given intravenously 100 mg./Kg. of rifomycin B twice a day, six days per week for four weeks without evidence of toxicity. The average body weights of these animals remained constant. Liver and kidney function tests and complete blood counts did not differ from predosing observations. On the basis of these results, the treatment of the 2 dogs will continue.

TESTS FOR PHARMACOLOGICAL ACTIVITY

Blood Pressure and Respiration. DOGS. Intravenous rifomycin B injection up to 100 mg./Kg. in 4 dogs under anesthesia (pentobarbital sodium 35 mg./Kg.) had no adverse effect on blood pressure and respiration. Successive intravenous doses of 50 mg./Kg. repeated four times at 20 to 30 minute intervals did not produce any significant alteration in the blood pressure or respiration of 3 dogs under anaesthesia (fig. 1). Only after a single intravenous injection of 150 and 200 mg./Kg. of rifomycin B was a fall in the blood pressure observed. In 3 experimented dogs there was an average maximum fall of 75 mm. Hg reduced to 50 per cent within 15 to 25 minutes. Respiration was contemporaneously depressed.

CATS. The blood pressure and respiration data on cats anesthetized with chloralose (70 mg./Kg.) present a picture almost identical with that obtained on dogs. Also in this species significant changes have been observed after intravenous administration of very high doses of rifomycin B, hypotension following 150 and 200 mg./Kg. of the antibiotic.

Effect on Vasomotor Responses. Rifomycin B does not modify the response to epinephrine, norepinephrine, histamine, acetylcholine, faradic stimulation of the peripheral stump of vagus nerve, or common carotid occlusion (fig. 1).

The tests were made on dogs anesthetized with chloralose or pentobarbital and the doses of rifomycin B ranged from 25 to 100 mg./Kg. After administration of higher doses of the substance, the hypertensive response to common carotid occlusion was depressed or even completely prevented and the effects of injected epinephrine reduced.

Ganglionic Transmission. In cats anesthetized with chloralose, 70 mg./Kg., the

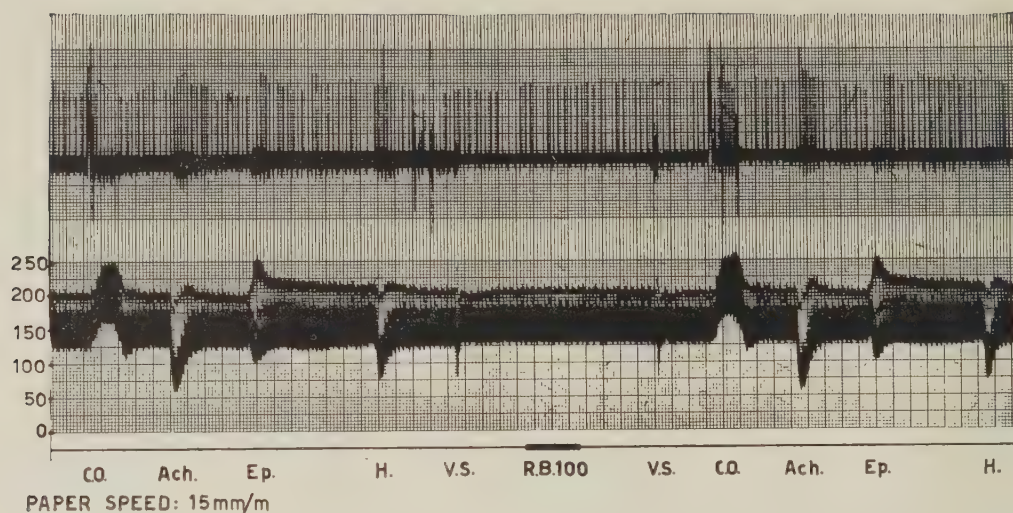


FIG. 1. Dog anesthetized with pentobarbital. Upper tracing: respiration. Lower tracing: blood pressure. (C.O. = right common carotid artery occlusion; Ach. = intravenous injection of acetylcholine, 2 µg./Kg.; Ep. = epinephrine, 2 µg./Kg.; H. = histamine, 2 µg./Kg.; V.S. = vagus nerve stimulation). At the signal rifomycin B, 100 mg./Kg., was injected intravenously.

contracture of nictitating membrane due to electrical stimulation of preganglionic sympathetic nerve was not modified after intravenous administration of rifomycin B in doses of 25, 50, and 75 mg./Kg.

Action on Isolated Tissues. Rifomycin B had essentially no effect on the isolated gut from the guinea pig or rat. In concentration from 10 to 50 µg./ml. in Tyrode solution, rifomycin B does not modify the usual responses of isolated rat ileum to acetylcholine and barium chloride nor the effect of histamine on similar preparation of guinea pig. Spontaneous contractility of isolated gut from the rabbit is also unchanged in the presence of 50 µg./ml. of rifomycin B.

In Langendorff isolated guinea pig heart doses of rifomycin B as high as 0.5 mg. do not produce significant changes in frequency and amplitude of cardiac beats.

On isolated guinea pig auricles no significant changes were observed after administration of rifomycin B in concentration up to 300 µg./ml. of Ringer-Webb solution.

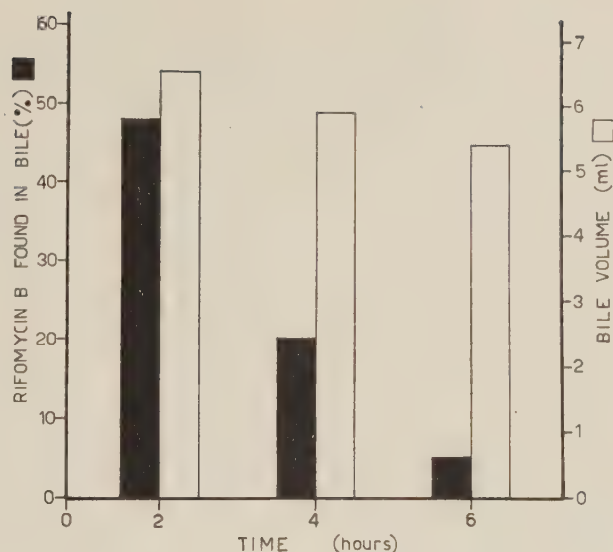
Blood Serum Levels. Single intramuscular doses of rifomycin B produced high average blood serum levels in 12 dogs, still within the first hour after administration. As shown in table II maximal microbiological activity is reached in the first

TABLE II

Rifomycin B Blood Serum Levels After a Single Intramuscular Dose in Dogs

Doses, mg./Kg.	Number of dogs	Average serum concentration in µg./ml. after					
		1 hour	3 hours	5 hours	6 hours	8 hours	24 hours
10	3	3.13	0.32	0.00	—	—	—
25	3	7.24	0.64	0.00	—	—	—
50	3	36.10	2.15	0.37	—	0.00	—
100	3	121.20	13.73	—	1.04	0.33	0.06

FIG. 2. Biliary excretion of rifomycin B in rats after subcutaneous administration (averages from 15 animals given 100 mg./Kg.). Bile volume measured in ml./10.



hour for any dose administered in a range from 10 to 100 mg./Kg. of body weight. Appreciable blood serum levels have been found three hours after the administration of 10 and 25 mg./Kg. and five and eight hours respectively after 50 and 100 mg./Kg. No appreciable blood serum levels were found after oral administration of doses as high as 200 mg./Kg. Intraduodenal administration of 200 mg./Kg. of rifomycin B produced very low serum levels in 2 anesthetized dogs.

In the liquor of three dogs, given 25 mg./Kg. intramuscularly of rifomycin B, no antibacterial activity was detected.

Bile and Urine Levels. Rifomycin B is concentrated in the bile of rats and dogs.

Fifteen male rats weighing 150 to 180 gr. received subcutaneously a single dose of 100 mg./Kg. of rifomycin B. Bile was collected every two hours for six hours

FIG. 3. Serum blood levels, biliary and urinary excretion of rifomycin B in dogs (averages from 3 dogs given 50 mg./Kg. intramuscularly).

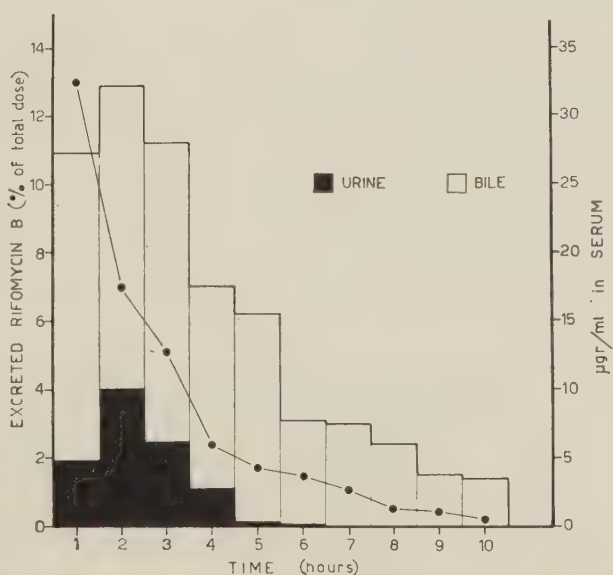


TABLE III

Percentages of Rifomycin B (50 mg./Kg.), Administered Intramuscularly, Found in Dogs' Bile and Urine Collected During a 10 Hour Period

Dog no.	Total dose, mg.	Rifomycin B found, %			
		Bile (microbiological assay)	Urine	Bile (spectrophotometric assay)	Urine
17	490	67.50	3.94	59.98	7.01
28	875	55.39	13.28	56.93	17.91
16	1150	57.10	15.74	62.60	16.40
Average		59.90	10.98	59.83	13.77

and microbiological determinations of antibiotic activity were performed on every individual sample. The results are shown in figure 2. It may be noted that, within two hours, 48 per cent of administered antibiotic is present in bile. Cumulative percentage of rifomycin B found in the six hour period averaged 74.8. During the experiment no sensible changes were evident in the volume of the excreted bile.

Three dogs anesthetized with pentobarbital sodium (36 mg./Kg. intravenously) were given 50 mg./Kg. intramuscularly of rifomycin B. Bile, blood, and urine samples were taken hourly for analysis. Blood levels were found to be more prolonged here than in nonanesthetized dogs treated with the same dose. Concentrations of rifomycin B as high as 8000 to 10,000 $\mu\text{g./ml.}$ were found in the bile collected within two and three hours from administration. Spectrophotometric analysis on every sample of urine and bile confirmed the presence of the antibiotic, approximately in the same concentration found by microbiological evaluation. Figure 3 shows the contemporaneous behavior of blood levels, biliary and urinary excretion of rifomycin B as a result of the pooled data obtained in 3 dogs.

Individual figure of total excreted rifomycin B, as per cent of the administered dose, is given in table II, as a result of both the microbiological and the spectrophotometric determinations.

The data obtained in dogs do not substantially differ from the ones previously reported in rats and confirm the peculiar tendency of rifomycin B to concentrate in the bile. The presence in the bile of so high concentration of rifomycin B appears to be of great interest in view of its therapeutic implications and will be further investigated.

Urinary excretion accounts for a minor quantity in respect to the elimination of rifomycin B. In anesthetized dogs, as shown in table III, no more than 17 per cent is excreted by kidneys.

TABLE IV

Urinary Excretion of Rifomycin B in Unanesthetized Dogs

Dose, mg./Kg., intramuscular	No. of dogs	Rifomycin B found in the urine excreted in 24 hours, %
10	3	5.3
25	3	4.7
50	3	7.4
100	3	5.6

TABLE V
Subcutaneous Irritation in Rats

Compound	Solution concentration, per cent	pH	Subcutaneous irritation					Total score
			Rat no.					
			1	2	3	4	5	
Rifomycin B sodium	25	7.0	1.2	1	1	1	1.2	9
Rifomycin B sodium	12.5	7.0	1	1	1	1.2	1	7
Rifomycin B sodium	6.2	7.0	0	1	0	1.2	0	4
Novobiocin sodium	25	8.0	1	1	1	1	1	5
Novobiocin sodium	12.5	7.7	0	1	1	1	1	4
Novobiocin sodium	6.2	7.5	0	1	0	1	0	2
Penicillin G sodium (1566 I.U./mg.)	25	7.0	0	1.3	1	1	1	7
Penicillin G sodium (1566 I.U./mg.)	12.5	7.1	1	1	1	1	1	5
Penicillin G sodium (1566 I.U./mg.)	6.2	7.2	0	0	0	2	0	2
Oleandomycin phosphate	25	5.3	1.3	1.3	1.3	1.3	1.3	20
Oleandomycin phosphate	12.5	5.3	1.3	1.3	1.2	1.3	1.3	19
Oleandomycin phosphate	6.2	5.3	1	1.3	1.2	1.3	1.2	15

In nonanaesthetized dogs, as shown in table IV, urinary excretion of rifomycin B appears to be minor than in anaesthetized dogs. Percentages of rifomycin B excreted with urine are rather constant within a dose range from 10 to 100 mg./Kg.

IRRITATION

Subcutaneous (Rat). Various concentrations of rifomycin B and other known antibiotics were administered to rats subcutaneously. A volume of 0.2 ml. of the various concentrations was injected into the back once a day for two days. Five animals were used for each concentration to be tested. An autopsy was performed on the third day and the degree of irritation has been recorded as follows: 0 = none; 1 = edema; 2 = hemorrhage; 3 = induration; and 4 = sterile abscess or necrotic damage.

As noted in table V, rifomycin B produces a certain degree of irritation also in the lower concentration tested. However, the degree of irritation produced by rifomycin B appeared to be markedly minor than that produced by oleandomycin phosphate and only slightly greater than that produced by sodium penicillin G.

Topical (Rabbit Eye). Two drops of various concentrations of rifomycin B were instilled into the conjunctival sacs of 3 rabbits each. This was repeated every two hours for a total of four instillations. Observations were made prior to each instillation of the drug and two hours after the final instillation.

No appreciable eye irritation was observed in any animal after instillation of 1, 10, and 25 per cent solutions of rifomycin B (pH 7.5). Except for a yellow discoloration of the conjunctiva no sign of irritation was evident in the treated rabbits, during the test period as well as in the days following the experiment.

SUMMARY

1. The pharmacological properties of rifomycin B have been studied in mice, rats, cats, and dogs.

2. The intravenous LD_{50} in mice was 2040 mg./Kg., in rats 1680 mg./Kg., and in dogs approximately 1200 mg./Kg. By intraperitoneal and subcutaneous routes mice and rats tolerated doses as high as 3000 mg./Kg.

3. Single daily intraperitoneal 2000 mg./Kg. and subcutaneous 500 and 2000 mg./Kg. doses in rats for 20 days produced no significant changes in growth rate, in any element of the complete blood count, and no pathological alteration was visible at gross and microscopic examination.

4. Daily intramuscular 250 mg./Kg. doses of rifomycin B in dogs for 44 days produced no deviation from the normality in liver or kidney function tests, blood clotting time, protein nitrogen levels, blood sugar levels, and complete blood counts.

5. Intravenous doses of 100 mg./Kg. twice a day were well tolerated by dogs for four weeks.

6. Rifomycin B given intravenously in dogs and cats produced essentially no changes in blood pressure and respiration; only after doses higher than 100 mg./Kg. was a slight hypotensive effect noted.

7. Rifomycin B did not modify either the vasomotor action of epinephrine, acetylcholine, or histamine, nor the effect of vagal stimulation upon the heart in cats and dogs.

8. Pharmacological evaluation indicated that rifomycin B has no significant ganglioplegic, antispasmodic, or anticonvulsant effect.

9. After intramuscular injection of single 50, 25, and 10 mg./Kg. doses, appreciable blood levels were found in dogs for five and three hours.

10. The antibiotic is concentrated in high degree in the bile of dogs and rats. During the first six hours after subcutaneous administration of 100 mg./Kg. doses, an average percentage of 74 per cent of the administered rifomycin B is excreted in the bile of rats. In dogs given 50 mg./Kg. of rifomycin B, about 60 per cent of the antibiotic was found in the bile collected in the 10 hours following the intramuscular administration.

11. Preliminary data indicate that rifomycin B does not cross the blood-brain barrier in normal dogs.

12. Rifomycin B seems to be poorly absorbed when administered orally and produces some local tissue irritation after subcutaneous and intramuscular injection.

ACKNOWLEDGMENTS

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Rifomycin. IV. Some Laboratory and Clinical Experiences with Rifomycin B

S. FÜRESZ AND R. SCOTTI

Research Laboratories, Lepetit S.p.A., Milano, Italy

Rifomycin B was isolated by Sensi et al¹ from *Streptomyces mediterranei*. The chemical, biological, and pharmacological properties of this antibiotic are reported in companion papers.²⁻⁴ The marked antibacterial activity, the low toxicity, and the promising results in experimental infections suggested further investigations concerning its clinical effectiveness.

In this paper, laboratory observations, namely, in vitro action on recently isolated *Staphylococcus* strains and blood levels after administration of rifomycin B in man, are reported. Further purpose of this paper is to present our first experiences as to the therapeutic efficacy of the antibiotic.

METHODS AND MATERIALS

Sensitivity Test. Fifty *Staphylococcus aureus* strains freshly isolated from different pathological sources were employed in this study. Forty of the strains were hemolytic and coagulase-positive, four failed to hemolyze, and six did not produce coagulase. The minimal inhibitory concentration of rifomycin B was determined by testing its antibacterial activity as compared with penicillin, novobiocin, tetracycline, streptomycin, erythromycin and oleandomycin. The agar streak dilution method with Penassay seed agar (Difco) was employed. For rifomycin B the pH of the medium was adjusted to 5.4 with 10 per cent of 1 M potassium dihydrogen phosphate solution. For the other antibiotics the pH value was maintained at 6.6, which is the best condition for their activity on agar. The lower pH value of the medium is required to assure the good diffusion of rifomycin B. A loopful of 1:10 dilution of a three hour broth culture of every strain has been used as inoculum. The minimal inhibiting concentration was the lowest concentration of antibiotic that prevented grossly visible growth after 24 hours of incubation.

Blood Levels. In order to determine the tolerance and the minimal amount that can give detectable levels in blood, increasing doses of rifomycin B were administered intramuscularly. Tests were carried out in adult patients with diseases not affecting absorption and elimination of the drug. Blood specimens were taken respectively one, two, and four or one, four, six, and nine hours after administration. The samples were promptly centrifuged and the serum removed. The determination was carried out on the same day. Blood levels of rifomycin B were determined with the plate assay technique. Nutrient agar (Difco) was used in two layers. Fifteen per cent of a 1 M potassium dihydrogen phosphate solution was added to the dissolved medium. Thus the pH decreases to 5.4. The test organism used was *Micrococcus pyogenes* var. *aureus* ATCC 6538, 299-P. The standard curve has been prepared by dissolving the working standard in phosphate buffer solution having a pH of 7.5. Further dilutions for the curve were made in normal human serum.

TABLE I

*Comparative Activity in Vitro of Rifomycin B and 5 Other Antibiotics
Against 50 Recently Isolated Strains of Staphylococcus aureus*

Antibiotics	Minimal inhibitory concentration, $\mu\text{g./ml.}^*$				
	<0.2	0.2-1	>1-5	>5-25	>25
Rifomycin B	49	1	0	0	0
Novobiocin	47	3	0	0	0
Tetracycline	0	36	1	4	9
Erythromycin	0	17	29	4	0
Oleandomycin	0	0	44	6	0
Penicillin	0	7	7	8	28

* These values for penicillin are expressed in units.

Clinical Trials. On the basis of the found blood levels, a daily dosage of 2.0 Gm. was established. This total dose was divided into two intramuscular injections of 1.0 Gm. each at 9 to 12 hours intervals. No treatment was carried out during the night. The daily dose for children was 30 mg./Kg. of body weight. All patients were carefully followed both clinically and by laboratory tests for the development of untoward effects to the drug. Even more careful attention was devoted to any liver damage. Following this treatment schedule, a total of 24 adult patients and 3 children were treated for a period of 1 to 10 days. In a few cases after prolonged therapy, doses were decreased to 1 Gm. daily.

RESULTS

In Vitro Sensitivity. The results of sensitivity tests are shown in table I. The growth of 49 out of 50 strains was inhibited by a concentration of rifomycin B lower than 0.2 $\mu\text{g./ml.}$ Only the activity of novobiocin was of the same order as rifomycin B, while the minimal inhibitory concentration of the other antibiotics studied has been remarkably higher for all strains tested. Furthermore, no cross

TABLE II

*Serum Concentration in Patients Receiving a Single Intramuscular
Dose of Rifomycin B (Expressed as Rifomycin B $\mu\text{g./ml.}$)*

Patient no.	Rifomycin B administered, mg.	Hours after administration		
		1	2	4
1	50	0.15	0.00	0.00
2	50	0.53	0.31	0.00
3	50	0.56	0.18	0.00
4	100	0.78	0.42	0.00
5	100	0.86	0.00	0.00
6	100	0.90	0.89	0.50
7	200	0.84	0.49	0.41
8	200	0.96	0.59	0.00
9	200	1.20	0.79	0.39
10	350	1.12	0.84	0.50
11	350	0.80	0.40	0.28
12	350	2.00	0.90	0.32

TABLE III

Serum Concentration in Patients Receiving a Single Intramuscular Dose of 500 mg. of Rifomycin B (Expressed as Rifomycin B μ g./ml.)

Patient no.	Hours after administration			
	1	4	6	9
1	2.35	0.26	0.10	—
2	0.90	0.36	0.24	—
3	3.50	0.47	0.33	—
4	2.60	0.29	0.12	—
5	5.80	0.46	0.26	—
6	4.25	1.08	0.65	—
7	3.35	0.61	0.40	—
8	4.80	—	0.36	0.28
9	4.30	—	1.76	0.52
10	0.96	—	0.17	0.00
11	2.70	—	0.18	0.00
12	0.96	—	0.00	0.00
Average	3.04	0.50	0.38	0.16

resistance between rifomycin B and the other five antibiotics has been found; strains inhibited only by 5 to 25 (or more) μ g./ml. of the other antibiotics were also sensitive to rifomycin B.

Absorption and Elimination. To investigate the absorption of rifomycin B, in a first trial the antibiotic was given orally. Not even a dose of 1 Gm. gave rise to detectable titers in blood. However, some rifomycin B was found in the urine showing that a minimal amount was still absorbed.

Rifomycin B was then given intramuscularly at increasing doses. No pain or

TABLE IV

Serum Concentration in Patients Receiving a Single Intramuscular Dose of 1.0 Gm. of Rifomycin B (Expressed as Rifomycin B μ g./ml.)

Patient no.	Hours after administration			
	1	4	6	9
1	5.40	0.72	0.45	—
2	3.70	0.60	0.45	—
3	3.60	0.62	0.39	—
4	8.40	0.53	0.27	—
5	9.80	3.70	2.45	—
6	7.20	2.60	1.38	—
7	7.10	0.68	0.42	—
8	4.20	1.55	1.55	—
9	16.80	1.25	0.38	—
10	6.70	0.84	0.40	—
11	12.00	1.30	0.58	—
12	6.00	0.49	0.15	—
13	9.60	—	0.64	0.35
14	12.00	—	0.62	0.35
15	6.50	—	0.36	0.15
16	11.00	—	0.68	0.28
17	10.70	—	0.84	0.30
Average	8.28	1.24	0.71	0.29

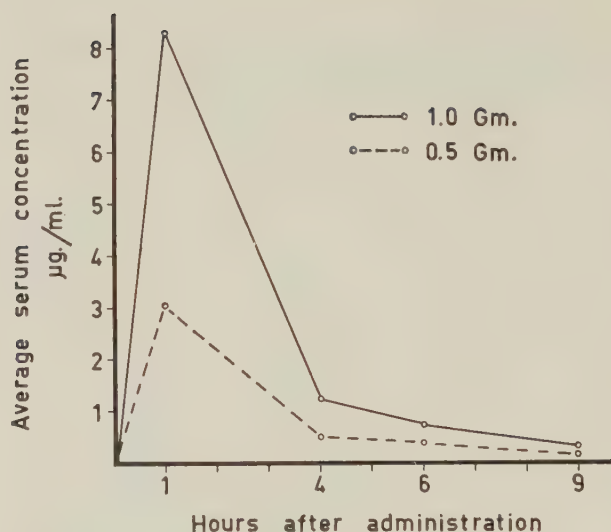


FIG. 1. Average serum concentrations of rifomycin B following a single intramuscular dose.

side effects were noted after a single dose of 10 and 25 mg. Following carefully the possible signs of intolerance, doses of 50, 100, 200, and 350 mg. were then given to 3 patients each. Blood serum levels obtained one, two, and four hours after administration of these doses are reported in table II. A single dose of 50 and 100 mg. gave rise to low titers of rifomycin B one hour after administration and traces of rifomycin B were detectable also after two hours. After administration of 200 and 350 mg. the blood levels in the first hours were only somewhat higher but four hours after administration there were still measurable quantities of antibiotic in the blood. The blood levels obtained after administration of 500 mg. and 1.0 Gm. respectively are reported in tables III and IV. It should be observed that, already in the levels of the first hour, the increase is remarkable. The serum titers are clearly higher than those obtained after a single dose of 350 mg. The differences are conspicuous in the blood specimens taken four, six, and nine hours after administration. While with 350 mg. the titers after four hours were very low, 500 mg. gave rise to measurable blood levels after six hours. After nine hours, there were still traces of rifomycin B in the blood serum. Another considerable increase in blood levels was noticed after administration of 1.0 Gm. of rifomycin B. A single dose of 1.0 Gm. indeed gave rise to an average of 8.26 µg./ml. after one hour. The titers obtained six and nine hours after administration are to be considered of therapeutic value. The average blood levels after administration of 500 mg. and 1.0 Gm. of rifomycin B are reported in figure 1.

Concerning the elimination of rifomycin B, it should be observed that even after intramuscular administration of 1.0 Gm. only a small amount was eliminated by the urinary tract. In the 24 hours after administration, only a small percentage of rifomycin B was detectable in urine. On the contrary, a good portion of the total amount of rifomycin B given intramuscularly was found in the stool. In 2 patients, blood and bile levels were determined simultaneously. Thirty minutes after administration of 1.0 Gm. of rifomycin B, 7200 and 1755 µg./ml. were found in the bile and 7.8 and 14.4 µg./ml. in the serum respectively. These data seem to indicate that rifomycin B is concentrated to a large extent in the bile. Rifomycin B

TABLE V

Results of Rifomycin B Therapy in 27 Patients with Various Types of Infections

Disease	Pt. no.	Antibiotics prior to rifomycin B	Duration rifomycin therapy, days	Total dose rifomycin B, Gm.	Response
Otitis media	1	None	3	6	Excellent
	2	None	6	1.2	Excellent
	3	None	4	0.8	Excellent
	4	Penicillin	4	3.2	Good
Dental abscess	5	Penicillin	4	8	Recovery by resolution
	6	None	4	8	Recovery by resolution
Furunculosis	7	None	3.5	7	Good
	8	None	5	10	Good
Proctitis, post-surgical diarrhea	9	Streptomycin, penicillin	6	12	Excellent
Mastitis, mammary abscess	10	Penicillin, streptomycin	6	12	Poor, healed after incision
	11	None	5.5	11	Recovery by resolution
	12	None	4.5	9	Recovery by resolution
	13	Penicillin, streptomycin	3.5	7	Good, healed after incision
	14	None	3.5	7	Recovery by resolution
Fever of unknown origin	15	Tetracycline, oleandomycin	10	15	Good
	16	Penicillin, tetracycline	8	16	Good
Pulmonary abscess	17	Novobiocin	6	12	Poor
	18	Novobiocin, chloramphenicol	7	12	Poor
Chronic osteomyelitis	19	None	7	13	Poor
	20	Several	4	8	Good
Gluteal abscess	21	None	3	6	Good
	22	Penicillin	4	8	Recovery by resolution
	23	Penicillin	1	2	Poor, healed after incision
Cholecystitis	24	Tetracycline	5	10	Good
	25	None	3	6	Good
	26	Several	6	12	Excellent
Follicular tonsillitis	27	Tetracycline	3.5	7	Failure

was also detectable in milk and in the ascitic fluid, but it did not pass through the healthy meninges.

Therapeutic Efficacy. Clinical results are summarized in table V. One of the 4 patients of otitis media was an adult, the others were children. Patients 1 and 3 had otitis showing remarkable inflammation of the tympanic membrane without suppuration. They healed completely respectively after two and four days of treatment. In patient 2 paracentesis before treatment gave rise to abundant purulent drainage. Fever decreased after two days of treatment and at the end of therapy also the excretion disappeared completely. Patient 4 was a case of chronic, recidivant otitis without fever but with abundant suppuration. At the end of treatment, no pus was detectable but the inflammation of the tympanic membrane was not completely resolved. The 2 patients with dental abscess (patients 5 and 6) healed completely. Patient 5 had a temperature of 40 C., patient 6 of 39 C. Both became afebrile after 2 Gm. of rifomycin B and the signs of inflammation and swelling rapidly disappeared.

One of the 2 cases of furunculosis, patient 7, had enormous confluent deep

furuncles with large infiltration all around. The signs of inflammation subsided after three injections of rifomycin B and 24 hours later the furuncles opened spontaneously and healed rapidly. *Staph. aureus* was cultured from the pus before and after treatment. Both strains were sensitive to rifomycin B. The other patient (8) was affected by Hodgkin's disease. He had many furuncles for 10 months on the whole body. After five days of treatment, the furuncles healed promptly and in two months of observation no new ones appeared. The *Staph. aureus* strains isolated before the treatment and on the third day of therapy were sensitive to rifomycin B. Patient 9 has had surgical treatment for anal sinus. He had colitis for many years. Eight days after the operation, diarrhea and fever (38–39 C.) appeared. Treatment with penicillin and streptomycin gave no results. Stools became normal after four days of treatment with rifomycin B and fever disappeared two days later.

Three of the 5 patients with mastitis (patients 11, 12, and 14) were treated in the earliest stage, i.e., within 24 hours after the first signs of illness when inflammation, pain, and fever were observed. They became afebrile after administration of 3 to 4 Gm. of rifomycin B. Rapid remission of signs of inflammation was noted. the patients were healed by complete resolution in four to five days. The other 2 patients (10 and 13) were treated with rifomycin B after other antibiotics had been given without any result and when the inflammatory process had reached the suppurative stage. Both patients become afebrile respectively after six and three days of treatment and symptoms showed a rapid diminution with limitation of the infiltrated area of the breast. In 1 of the patients (10) an exploratory puncture was undertaken at the end of the treatment and some millilitres of pus were obtained. From this pus, a rifomycin sensitive *Staph. aureus* strain was cultured. Three days later the abscess was incised and no *Staph. aureus* or other bacteria were obtained from the pus. The other patient (13) was also operated on but only a small incision was necessary. The *Staph. aureus* strain cultured from the pus was also sensitive to rifomycin B and the strain obtained some days later from the wound showed the same sensitivity.

Patient 15 was hospitalized after some weeks of continuous temperature of 38 to 39 C. He was suffering pain at the right leg and foot. The diagnosis of radiculitis of unknown origin was established. All attempts to isolate a bacterial agent (repeated blood and marrow cultures) had no success. Fever persisted after tetracycline and oleandomycin treatment. In spite of the doubtful diagnosis, rifomycin B therapy was undertaken. The patient became afebrile after 10 days of treatment. (In the last five days only, 1.0 Gm. was given.) Pain decreased remarkably. Fifteen days later, fever reappeared but after a new cycle of rifomycin B (1.0 Gm./6 days) the patient became again afebrile. During two months of observation the temperature remained normal and also the signs of radiculitis disappeared gradually. The other patient (16) had fever for four weeks. The temperature rose to 38–39 C. in the afternoon. Several clinical and laboratory investigations failed to establish the origin of the fever. General conditions became rapidly worse. Various antibiotics given at home and after hospitalization gave no result. Rifomycin treatment was then undertaken, but the elevated temperature persisted for several days. The patient became afebrile only eight days later when prednisone was added to rifomycin treatment. The 3 patients with lung abscess (17, 18, and 19) showed only poor and transitory improvement after rifomycin B therapy. One of them became

afebrile but fever reappeared some days later. All patients immediately after the beginning of the treatment showed remarkable decrease in cough and sputum but this improvement gradually subsided.

The patient with chronic osteomyelitis of coxofemoral joint (20) had surgical operation 15 years before. Occasionally discharging chronic sinuses were present. Different antibiotics were used with doubtful success. During one of the acute episodes rifomycin B was administered and four days later symptoms regressed. Definitive evaluation is not yet possible.

One case of gluteal abscess (patient 21) had an operation and a large drainage was performed. Abundant pus edema and inflammation of the surrounding tissues persisted many weeks after the operation. *Staph. aureus* was cultured from the pus. The strain was sensitive to rifomycin B. Topical treatment was undertaken using a 0.5 per cent water solution of rifomycin B. The response was immediate. After the first applications, signs of inflammation on the edges diminished and drainage decreased. Intramuscular administration of rifomycin B was then started, because the edema of the whole gluteal region persisted and the patient had also fever. Three days later the temperature turned downward and the wound condition improved. Patient 22 was taken to the hospital with an enormous abscess on the back following intramuscular injection. The entire right gluteal region was infiltrated causing severe pain and temperature about 39–40 C. No pus was obtained by exploratory puncture. Following treatment with rifomycin B, the patient became afebrile within 24 hours. Pain and inflammation showed rapid remission. After total dosage of 8 Gm. of rifomycin B, there was loss of redness and induration. The patient healed completely. The third patient with gluteal abscess (23) was taken to the hospital with a large fluctuating abscess on back and with 39 C. temperature. He became afebrile after two injections of rifomycin B but the next day he was operated and healing followed after drainage.

Patient 24 was hospitalized with marked abdominal pain in the right upper quadrant, fever (38–39 C.) and slight jaundice. Treatment with tetracycline gave only a transitory improvement. A second trial with tetracycline had no result. Rifomycin B was then administered. Pain rapidly decreased but fever persisted for some days and subsided after five days of treatment. Nine days later he was healed and discharged. No further data are available. The 2 remaining cases of this group had both subchronic cholecystitis. The first one (25) suffered for two months of typical gallbladder attacks without biliary obstruction. During the intervals he noted pain on the right upper quadrant. Rifomycin B was given for a three day period and since then no further attacks occurred. During three months of observation, the patient showed no signs of illness. The other patient (26) suffered for eight months from repeated gallbladder attacks with nausea, vomiting, transitory jaundice, and fever. He was treated at home and in another hospital, but he showed no improvement. Rifomycin B treatment was undertaken and, after a total dose of 12 Gm., the patient felt better, was afebrile and was released. During one month of observation at home, he was asymptomatic and gained 4 Kg. in body weight.

The last patient (27) can be considered as a drug failure. After tetracycline therapy, which gave no result, rifomycin B was administered for 3.5 days but neither the local conditions improved nor the temperature decreased. The patient became afebrile after penicillin, streptomycin, and prednisone.

No side reactions were observed during rifomycin B therapy. On the contrary, the patients felt a remarkable improvement of the general conditions in the first days of treatment. The sense of illness rapidly diminished even when fever still persisted. Local pain after the injections was occasionally observed. In the earlier stage of treatment, after two or four injections, pain was meaningless but after a longer period of therapy some individuals have mentioned severe backache. A great personal variability was noted. Several patients had no pain after six or seven days of therapy. However, induration without signs of inflammation at side of the injections was noted but pain and local symptoms disappeared rapidly when treatment was stopped.

Our preliminary clinical experience seems to indicate that rifomycin B offers good promises in the treatment of micrococcal infections and perhaps in other types of infections, particularly those regarding the gallbladder inflammation. These results suggest that further clinical investigations on rifomycin B are justified.

SUMMARY

1. The in vitro sensitivity of 50 freshly isolated staphylococci was determined. All strains tested were sensitive to rifomycin B.

2. Rifomycin B was not absorbed after oral administration. One hour after intramuscular administration of 500 mg. and 1.0 Gm., average values respectively of 3.04 and 8.28 $\mu\text{g.}/\text{ml.}$ were found in blood serum. After six and nine hours, titers of the therapeutic value were still detectable. Rifomycin B is highly concentrated in the bile, diffuses into the ascitic fluid and in the milk, but does not pass through the healthy meninges.

3. Twenty-seven patients were treated with rifomycin B. The types of infections included: otitis media, dental abscess, furunculosis, proctitis, postsurgical diarrhea, mastitis or mammary abscess, fever of unknown origin, pulmonary abscess, chronic osteomyelitis, cholecystitis, gluteal abscess, and follicular tonsillitis.

A daily dosage of 2.0 Gm. was established. This total dose was divided into two intramuscular injections of 1.0 Gm. at 1 to 12 hour intervals. The therapeutic effect of rifomycin B was definite and convincing. The majority of patients became afebrile within 24 to 72 hours and local inflammatory and infiltrative processes showed a rapid regression. In the cases of pulmonary abscess the therapeutic effect was only transitory. The case of follicular tonsillitis must be considered as a "drug failure." No side reactions were observed during the therapy. Only in few cases was pain at the site of the injection observed after a prolonged treatment.

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Paromomycin: Experimental Antibacterial Activity

MYRON W. FISHER, MARY C. MANNING, LEOLA A. GAGLIARDI,
MARJORIE R. GAETZ, AND ARVID L. ERLANDSON

Research Laboratories, Parke, Davis & Company, Detroit, Mich.

A brief account of the experimental antibacterial properties of paromomycin* in vitro and in vivo has been given in a preliminary publication.¹ It is the purpose of this report to provide an expanded, detailed account of the activity of this antibiotic against various bacteria. Since resistance and cross-resistance data on paromomycin have been published elsewhere,² these aspects will not be included here.

All of the studies described herein were performed with the sulfate salt of paromomycin, representing approximately 75 per cent antibiotic base. The data pertaining to paromomycin concentrations will be based on this sulfate salt.

MATERIALS AND METHODS

Bacteriostatic Tests In Vitro. Stock solutions of paromomycin were autosterilized as high concentrations in 95 per cent ethanol. All tests were conducted by the broth dilution method, using Trypticase soy broth (BBL) with the following exceptions: *Clostridium perfringens* was tested in Brewer's thioglycollate (Difco), and the species of *Mycobacterium* were tested in a defined medium³ containing 10 per cent (v/v) bovine serum. Beginning with levels of 100 or 20 µg./ml., serial twofold decrements of paromomycin were set up in 5 ml. volumes in aluminum-capped tubes. Each tube was inoculated with 0.1 ml. of a 10^{-5} dilution of broth growth taken at the early maximum growth phase. Minimum total inhibition concentrations were estimated by visual inspection after 24 to 48 hours incubation at 37 C. for the non-mycobacterial species, and after seven days for the mycobacteria. The results of these determinations, which were derived from at least two replicate tests, are given in table I.

Bactericidal Tests In Vitro. ACTIVITY AGAINST KLEBSIELLA PNEUMONIAE. Four flasks, each containing 15 ml. of Trypticase soy broth plus 10 per cent bovine serum, were inoculated with 1.0 ml. of six hour broth growth of *Klebsiella pneumoniae*, strain AD, to provide a final concentration of 5×10^6 cells/ml. Each flask was incubated at 37 C. for one hour to institute logarithmic phase growth, and then paromomycin was added to provide levels of 66.7, 33.3, 6.7, and 3.3 µg./ml./flask. Incubation was continued and aliquots were removed at 15 to 30 minute intervals for plate counts. Preliminary tests indicated that there was a negligible effect from paromomycin carry-over in the agar subcultures. The effect of paromomycin on viability under these conditions is illustrated in figure 1.

ACTIVITY AGAINST A HEAVY MIXED BACTERIAL SUSPENSION. The following bacteria were employed in these tests: *Staphylococcus aureus*, strains S-744 and S-758 (both strains were resistant to streptomycin, tetracyclines, erythromycin, and penicillin), *Streptococcus pyogenes*, strains C203 and PD-04715, *Escherichia coli*, strains MGH-2 and MGH-3, *Proteus vulgaris*, strains 1810 and UC232, *Proteus*

* The trade name of Parke, Davis & Co. for paromomycin is Humatin.

TABLE I
Antibacterial Inhibitory Activity of Paromomycin in Vitro

Organism	Strain	Minimal 100 per cent inhibitory conc., $\mu\text{g./ml.}$
Gram-Positive Bacteria		
<i>Clostridium perfringens</i>	PD-02122	>100.0
<i>Corynebacterium diphtheriae</i>	PD-036	0.4
<i>Diplococcus pneumoniae</i>	SVI	100.0
<i>Diplococcus pneumoniae</i>	PD-04385	100.0
<i>Staphylococcus aureus</i>	UC76	0.8
<i>Staphylococcus aureus</i>	Smith	0.8
<i>Staphylococcus aureus</i>	PD-04778	0.8
<i>Staphylococcus aureus</i>	PD-02482	1.6
<i>Staphylococcus aureus</i>	PD-04985	3.1
<i>Staphylococcus aureus</i>	PD-04988	3.1
<i>Staphylococcus aureus</i> , 80/81	TU-12404	1.6
<i>Staphylococcus aureus</i> , 80/81	Padgett	0.8
<i>Staphylococcus aureus</i> , 80/81	Schneider	0.8
<i>Streptococcus faecalis</i>	MGH-1	6.3
<i>Streptococcus faecalis</i>	MGH-2	3.1
<i>Streptococcus pyogenes</i>	C203	50.0
<i>Streptococcus pyogenes</i>	PD-04472	25.0
<i>Streptococcus salivarius</i>	PD-04150	50.0
Gram-Negative Bacteria		
<i>Aerobacter aerogenes</i>	PD-0126	6.3
<i>Aerobacter aerogenes</i>	MGH-1	3.1
<i>Aerobacter aerogenes</i>	MGH-3	3.1
<i>Aerobacter aerogenes</i>	MGH-6	0.8
<i>Brucella suis</i>	H-1772	3.1
<i>Escherichia coli</i>	PD-04289	6.3
<i>Escherichia coli</i>	PD-04863	6.3
<i>Escherichia coli</i>	055:B5	3.1
<i>Escherichia coli</i>	086:B7	6.3
<i>Escherichia coli</i>	0111:B4	3.1
<i>Escherichia coli</i>	0119:B14	6.3
<i>Escherichia coli</i>	0125:B15	6.3
<i>Escherichia coli</i>	0127:B8	3.1
<i>Klebsiella pneumoniae</i>	AD	1.6
<i>Klebsiella pneumoniae</i>	PD-04544	1.6
<i>Klebsiella pneumoniae</i>	MGH-1	6.3
<i>Klebsiella pneumoniae</i>	MGH-2	3.1
<i>Neisseria catarrhalis</i>	PD-03447	0.8
<i>Paracolobactrum arizonae</i>	C14	6.3
<i>Paracolobactrum arizonae</i>	C15	6.3
<i>Paracolobactrum arizonae</i>	C16	12.5
<i>Paracolobactrum aerogenoides</i>	C202	3.1
<i>Paracolobactrum intermedium</i>	C401	3.1
<i>Paracolobactrum intermedium</i>	C402	3.1
<i>Paracolobactrum intermedium</i>	C424	3.1
<i>Paracolon species</i> , "2911"	C106	6.3
<i>Pasteurella multocida</i>	PD-02855	6.3
<i>Proteus mirabilis</i>	MGH-1	6.3
<i>Proteus mirabilis</i>	MGH-2	6.3
<i>Proteus mirabilis</i>	MGH-3	25.0
<i>Proteus mirabilis</i>	MGH-4	25.0
<i>Proteus mirabilis</i>	MGH-5	12.5
<i>Proteus mirabilis</i>	MGH-6	50.0
<i>Proteus mirabilis</i>	SK-8552	12.5

Table I Continued on Page 295

TABLE I (Continued)

Antibacterial Inhibitory Activity of Paromomycin *in Vitro*

Organism	Strain	Minimal 100 per cent inhibitory conc., µg./ml.
Gram-Negative Bacteria (Continued)		
<i>Proteus mirabilis</i>	SK-8631	50.0
<i>Proteus mirabilis</i>	SK-8651	25.0
<i>Proteus mirabilis</i>	SK-8369	12.5
<i>Proteus morganii</i>	SK-7015	3.1
<i>Proteus morganii</i>	SK-7127	3.1
<i>Proteus rettgeri</i>	MGH-1	6.3
<i>Proteus vulgaris</i>	PD-04736	3.1
<i>Proteus vulgaris</i>	1810	12.5
<i>Proteus vulgaris</i>	UC232	1.6
<i>Proteus vulgaris</i>	MGH-19	6.3
<i>Proteus vulgaris</i>	MGH-23	0.2
<i>Proteus vulgaris</i>	MGH-24	50.0
<i>Proteus vulgaris</i>	MGH-26	6.3
<i>Proteus vulgaris</i>	MGH-29	6.3
<i>Proteus vulgaris</i>	MGH-50	25.0
<i>Proteus vulgaris</i>	MGH-53	12.5
<i>Proteus vulgaris</i>	MGH-142	3.1
<i>Pseudomonas aeruginosa</i>	Duke	25.0
<i>Pseudomonas aeruginosa</i>	PD-01925	50.0
<i>Pseudomonas aeruginosa</i>	MGH-28	> 100.0
<i>Pseudomonas aeruginosa</i>	MGH-68	> 100.0
<i>Salmonella paratyphi</i>	PD-02156	1.6
<i>Salmonella schottmuelleri</i>	PD-01180	12.5
<i>Salmonella typhimurium</i>	V31	12.5
<i>Salmonella typhimurium</i>	Texas	25.0
<i>Salmonella typhimurium</i>	R1A-1	25.0
<i>Salmonella typhosa</i>	PD-02481	6.3
<i>Shigella dysenteriae</i>	PD-01339	12.5
<i>Shigella flexneri</i> , type 2a	B908	12.5
<i>Shigella flexneri</i> , type 2a	B963	25.0
<i>Shigella flexneri</i> , type 3	B1013	12.5
<i>Shigella flexneri</i> , type 3	B1070	12.5
<i>Shigella flexneri</i> , type 3	B1084	25.0
<i>Shigella flexneri</i> , type 3	B1093	12.5
<i>Shigella flexneri</i> , type 5	B1302	12.5
<i>Shigella flexneri</i> , type 5	B1331	25.0
<i>Shigella flexneri</i> , type 5	B1302	25.0
<i>Shigella flexneri</i> , type 6	B1404	12.5
<i>Shigella flexneri</i> , type 6	B1488	25.0
<i>Shigella flexneri</i> , type 6	B1447	25.0
<i>Shigella sonnei</i>	B2571	12.5
<i>Shigella sonnei</i>	B2598	25.0
<i>Shigella sonnei</i>	B2599	12.5
<i>Shigella sonnei</i>	PD-04628	12.5
<i>Vibrio comma</i>	PD-04643	25.0
Acid-Fast Bacilli		
<i>Mycobacterium avium</i>	Kirchberg	0.63
<i>Mycobacterium avium</i>	MC4186	20.0
<i>Mycobacterium fortuitum</i>	ATCC-6841	20.0
<i>Mycobacterium microti</i>	NCTC-5676	20.0
<i>Mycobacterium smegmatis</i>	PD-01328	0.3
<i>Mycobacterium bovis</i>	UM374	0.1
<i>Mycobacterium bovis</i>	UM375	0.1
<i>Mycobacterium tuberculosis</i>	H ₃₇ Rv	0.1
<i>Mycobacterium tuberculosis</i>	MC933	0.3
<i>Mycobacterium tuberculosis</i>	MC969	0.1
<i>Mycobacterium tuberculosis</i>	MC811	0.3
<i>Mycobacterium tuberculosis</i>	MC892	0.2

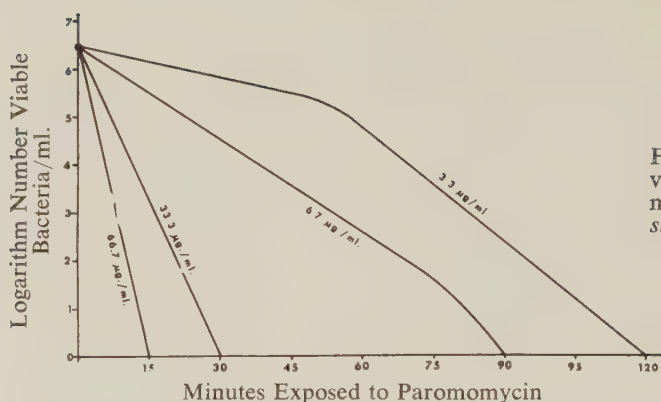


FIG. 1. Bactericidal activity of various concentrations of paromomycin in vitro against *Klebsiella pneumoniae*.

mirabilis, strains MGH-3 and MGH-6, and *Pseudomonas aeruginosa*, strains MGH-2 and MGH-6. Each organism was inoculated on two agar slants, incubated for 24 hours, and the growth removed and pooled to provide a dense, mixed suspension of about 10^{10} cells/ml. One volume of this suspension was added to nine volumes of 10 per cent serum-Trypticase soy broth, and paromomycin was added to aliquots of the broth suspension in final concentrations of 50 through 500 $\mu\text{g./ml.}$ Incubation was continued at 37 C., and each preparation was sampled at hour intervals for subculture on Trypticase soy agar and E.M.B. agar plates. In this manner an attempt was made to determine when each concentration of paromomycin effected complete sterilization and when each species was killed. The results of this experiment are given in table II.

ACTIVITY AGAINST BACTERIA IN FECES. Fecal pellets were freshly collected from mice and triturated to about 50 per cent concentration in saline. Paromomycin was then added to a final concentration of 1 mg./ml. and the mixture was held at 37 C. At 30 minute intervals, samples were subcultured as in the previously described test to provide an estimate of the rate of sterilization of aerobic bacteria. The results of this test are given in the section on results.

Antibacterial Tests in Experimental Nonmycobacterial Infections of Mice. Albino Swiss mice, 18 to 22 Gm., either sex, were injected intraperitoneally with approximately 100 LD₅₀ of bacteria derived from six hour growth in Trypticase soy broth. The following species were employed: *Staph. aureus*, *Str. pyogenes*, *E. coli*, *K. pneumoniae*, *Paracolonobacterium arizonae*, *P. mirabilis*, *P. vulgaris*, *Ps. aeruginosa*, *Sal-*

TABLE II

Bactericidal Activity of Paromomycin Against a Heavy Suspension of a Bacterial Mixture

Paromomycin, $\mu\text{g./ml.}$	Hours required for killing				
	<i>Str.</i> <i>pyogenes</i>	<i>Staph.</i> <i>aureus</i>	<i>E. coli</i>	<i>Proteus</i> species	<i>Ps.</i> <i>aeruginosa</i>
50	6.5	1.5	<0.5	>7	>7
100	5.5	1.5	<0.5	4.0	>7
200	5.0	1.5	<0.5	1.5	>7
500	1.5	1.5	<0.5	1.0	>7

TABLE III

Activity of Paromomycin in Experimental Bacterial Infections of Mice

Infecting organism	Route of treatment	Regimen	Approximate 50% effective dose, mg./Kg./day
<i>Staphylococcus aureus</i> , Smith	Subcutaneous	Single dose	1.2
<i>Staphylococcus aureus</i> , Smith	Subcutaneous	2 doses/day; 3 days	1.4
<i>Staphylococcus aureus</i> , Smith	Oral	Single dose	27.0
<i>Staphylococcus aureus</i> , Smith	Oral	2 doses/day; 3 days	86.0
<i>Staphylococcus aureus</i> , UC76	Subcutaneous	Single dose	1.2
<i>Streptococcus pyogenes</i> , C203	Subcutaneous	Single dose	78.0
<i>Klebsiella pneumoniae</i> , AD	Subcutaneous	Single dose	4.0
<i>Klebsiella pneumoniae</i> , AD	Subcutaneous	2 doses/day; 3 days	4.0
<i>Klebsiella pneumoniae</i> , AD	Oral	2 doses/day; 3 days	200.0
<i>Klebsiella pneumoniae</i> , MGH-1	Subcutaneous	Single dose	4.0
<i>Escherichia coli</i> , Vogel	Subcutaneous	Single dose	8.4
<i>Escherichia coli</i> , MGH-2	Subcutaneous	Single dose	9.2
<i>Escherichia coli</i> , 055:B5*	Subcutaneous	Single dose	5.5
<i>Escherichia coli</i> , 0111:B4*	Subcutaneous	Single dose	6.3
<i>Escherichia coli</i> , 0119:B14*	Subcutaneous	Single dose	8.0
<i>Paracolobactrum arizonae</i> , C14	Subcutaneous	Single dose	7.3
<i>Paracolobactrum arizonae</i> , C15	Subcutaneous	Single dose	7.0
<i>Paracolobactrum arizonae</i> , C16	Subcutaneous	Single dose	5.8
<i>Proteus mirabilis</i> , MGH-1	Subcutaneous	Single dose	5.0
<i>Proteus mirabilis</i> , MGH-1	Oral	Single dose	90.0
<i>Proteus mirabilis</i> , MGH-3	Subcutaneous	Single dose	7.3
<i>Proteus vulgaris</i> , 1810	Subcutaneous	Single dose	7.2
<i>Pseudomonas aeruginosa</i> , MGH-28	Subcutaneous	2 doses/day; 3 days	>250.0
<i>Pseudomonas aeruginosa</i> , MGH-3	Subcutaneous	Single dose	100.0
<i>Salmonella typhimurium</i> , V31	Subcutaneous	2 doses/day; 3 days	150.0
<i>Shigella flexneri</i> , type 3, C1084	Subcutaneous	Single dose	17.5
<i>Shigella flexneri</i> , type 3, C1070	Subcutaneous	Single dose	17.0
<i>Shigella flexneri</i> , type 3, C1093	Subcutaneous	Single dose	14.8
<i>Shigella flexneri</i> , type 6, C1447	Subcutaneous	Single dose	8.5
<i>Shigella sonnei</i> , C2571	Subcutaneous	Single dose	8.5

* Enteropathogens.

monella typhimurium, *Shigella flexneri*, and *Shigella sonnei*. Hog gastric mucin, 5 per cent, was used as an adjuvant for all bacteria except the strains of *Str. pyogenes*, *K. pneumoniae*, and *Sal. typhimurium*. Peroral or subcutaneous treatment with paromomycin was given promptly after challenge in single or divided doses. When two daily doses were given, the first dose was administered at about 9 a.m. and the second dose at 3 p.m. Graded levels of paromomycin were employed throughout to permit the estimation of the 50 per cent effective dose by the Miller-Tainter method.⁴ Groups of 15 to 20 mice were used for each regimen, all experiments were performed in replicate, and final data on survival were taken at periods representing about three times the median survival time of untreated control groups. The results of these experiments are given in table III.

Bactericidal Activity In Vivo. This experiment was performed with *K. pneumoniae*, strain AD, the same strain that was used for the bactericidal test in vitro. Mice were injected intraperitoneally with about 10,000 LD₅₀, a dose calculated from preliminary experiments to induce a prompt septicemia and a level of nearly 10 million bacteria per ml. of blood in one hour. Therefore, one hour after challenge, mice were given a single subcutaneous dose of paromomycin at 2.5, 5.0, 25.0, and 50.0 mg./Kg., using 2 mice per dose level. At frequent intervals prior to

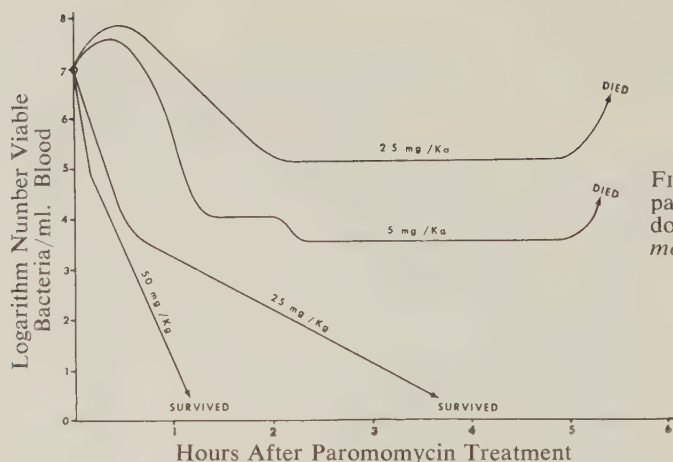


FIG. 2. Bactericidal activity of paromomycin in vivo at various dose levels against *Klebsiella pneumoniae*.

and subsequent to challenge, a blood sample was taken from each mouse with a hemocytometer pipette, calibrated to remove 0.0025 ml. of blood from tail tip amputation. These blood samples were diluted in saline for plate count determinations by the Miles-Misra method.⁵ The results of this experiment are shown in figure 2.

Activity of Paromomycin in Experimental Tuberculosis. These studies were performed in four laboratories: originally by Dr. Guy P. Youmans at Northwestern University Medical School, and subsequently by Dr. William Steenken, Jr., at the Trudeau Laboratory, Dr. Alfred G. Karlson at the Mayo Clinic, and at the Parke, Davis laboratories. The H₃₇Rv strain of *Mycobacterium tuberculosis* was used in all experiments, and paromomycin was administered subcutaneously. Tests were conducted in experimentally infected mice and guinea pigs, and streptomycin was employed, where possible, as a reference antibiotic.

EXPERIMENTS WITH MICE. In the experiments performed by Dr. Youmans, 18 to 22 Gm. Strong A mice were injected intravenously with 1.0 mg. (wet weight) of *Myco. tuberculosis* H₃₇Rv, and treatment was begun on the same day. In a representative experiment, a single daily dose of paromomycin was given to groups of 10 mice at levels of 50 through 400 mg./Kg./day for 14 consecutive days. In another experiment, dosages of 100 through 400 mg./Kg./day were administered in two divided daily doses for 10 days to groups of 19 or 20 mice. The results of these experiments are given in the upper section of table IV.

An additional experiment was performed by Dr. William Steenken, Jr. He employed CF-1 mice, 20 Gm., challenged intravenously with 0.5 mg. wet weight *Myco. tuberculosis* H₃₇Rv. Paromomycin dosages of 50 through 200 mg./Kg./day were given to groups of 9 to 11 mice starting one day after challenge. As in Dr. Youmans' study, paromomycin was given in one or two divided daily doses, but this treatment was maintained for 16 days. The results of this experiment are given in the center section of table IV.

A third experiment was performed at Parke, Davis, wherein CF-1 mice, 16 to 18 Gm., were injected intravenously with 1.0 mg. wet weight of H₃₇Rv. Paromomycin treatment, 10 mice per regimen, was begun on the day of challenge, employing dosages of 50 through 200 mg./Kg./day. The antibiotic was given as a single daily

TABLE IV
Activity of Paromomycin in Experimental Murine Tuberculosis
(All Treatment Subcutaneous)

Laboratory	Antibiotic	Mg./Kg./day	Doses/day	Days treated	Number of mice	ST ₅₀ * days
Northwestern†	None	—	—	—	10	13.5
	Streptomycin	50	1	14	10	24
	Paromomycin	50	1	14	10	14.5
	Paromomycin	100	1	14	10	19
	Paromomycin	200	1	14	10	30
	Paromomycin	400	1	14	10	Toxic
	Streptomycin	50	2	10	20	35
	Paromomycin	100	2	10	20	42
	Paromomycin	200	2	10	19	59
	Paromomycin	300	2	10	20	56
	Paromomycin	400	2	10	19	68
	None	—	—	—	9	9
Trudeau‡	Streptomycin	100	1	16	5	60
	Paromomycin	50	1	16	10	9
	Paromomycin	100	1	16	9	14
	Paromomycin	200	1	16	9	22
	Paromomycin	50	2	16	10	11
	Paromomycin	100	2	16	10	13
	Paromomycin	200	2	16	11	21
	None	—	—	—	10	9
Parke, Davis	Streptomycin	50	2	11	10	17
	Paromomycin	50	1	11	10	17
	Paromomycin	100	1	11	10	18
	Paromomycin	200	1	11	10	22
	Paromomycin	50	2	11	10	10
	Paromomycin	100	2	11	10	17
	Paromomycin	200	2	11	10	23
	None	—	—	—	10	9

* Median survival time.

† Performed by Dr. Guy P. Youmans, Northwestern University Medical School.

‡ Performed by Dr. William Steenken, Jr., Trudeau Laboratory.

dose or two divided daily doses for a total of 11 days. The results of these tests are given in the bottom section of table IV.

EXPERIMENTS WITH GUINEA PIGS. In a study carried out by Dr. Steenken, guinea pigs were injected intracardially with 0.1 mg. wet weight of *Myco. tuberculosis* H₃₇Rv. Employing 5 animals per regimen, and starting 7 days after challenge, he treated one group with a single daily dose of paromomycin at 50, 100, and 200 mg./Kg., and a second group was given the same amounts in 2 divided daily doses. This treatment was continued for 48 days, and the surviving guinea pigs were sacrificed 42 days after treatment was stopped (or 97 days after the infection was induced). The extent of disease was estimated from the amount of gross pathology observed at the time of sacrifice, and the pertinent data are given in table V.

An additional trial of paromomycin in guinea pig tuberculosis was performed by Dr. Alfred G. Karlson. In this study, 26 male guinea pigs, weighing approximately 700 Gm. each, were injected intraperitoneally with 0.1 mg. of *Myco. tuberculosis* H₃₇Rv. Nineteen days later, 6 animals were sacrificed and were found to have well-established disease. At this time, 10 untreated controls were retained and 10 guinea pigs were given a single daily dose of 143 mg. paromomycin/Kg. Because some toxicity was noted, this regimen was changed after 10 days to a single daily dose of 72 mg./Kg., continued for 54 days. On the eighty-third day after challenge,

TABLE V

Activity of Paromomycin in Experimental Guinea Pig Tuberculosis*
(All Treatment Subcutaneous)

Antibiotic	Mg./Kg./day	Doses/day	ST ₅₀ † days	Average gross tuberculosis index (16 maximum)
None	—	—	22	9.6
Streptomycin	35	1	>97	7.4
Paromomycin	50	1	62	8.3
Paromomycin	100	1	>97	8.8
Paromomycin	200	1	18‡	8.5
Paromomycin	50	2	73	8.0
Paromomycin	100	2	>97	8.9
Paromomycin	200	2	18‡	Not obtainable

* Tests performed by Dr. William Steenken, Jr., Trudeau Laboratory.

† Median survival time.

‡ Probable toxic reaction.

which was the sixty-fourth day of paromomycin treatment, 8 surviving paromomycin-treated animals and 1 surviving control were sacrificed and examined for gross pathology. The results of this experiment are depicted in figure 3, wherein the number under each animal indicates the day of death postinfection, and a black bar represents the death of an animal before the experiment was terminated. (For a full description of this procedure, see the report of Feldman.⁶)

RESULTS

Antibacterial Spectrum of Paromomycin In Vitro. The data given in table I indicate consistently good activity of paromomycin against strains of *Corynebacterium diphtheriae*, *Staph. aureus*, *Streptococcus faecalis*, *Aerobacter aerogenes*, *Brucella suis*, *E. coli*, *K. pneumoniae*, *Neisseria catarrhalis*, paracolon bacilli, *Pasteurella multocida*, *Proteus morgani*, and *Proteus rettgeri*. Somewhat less consistent activity occurred against strains of *P. mirabilis*, *P. vulgaris*, and species of *Salmonella*. Moderate paromomycin activity was noted for *Vibria comma*, nonmammalian mycobac-

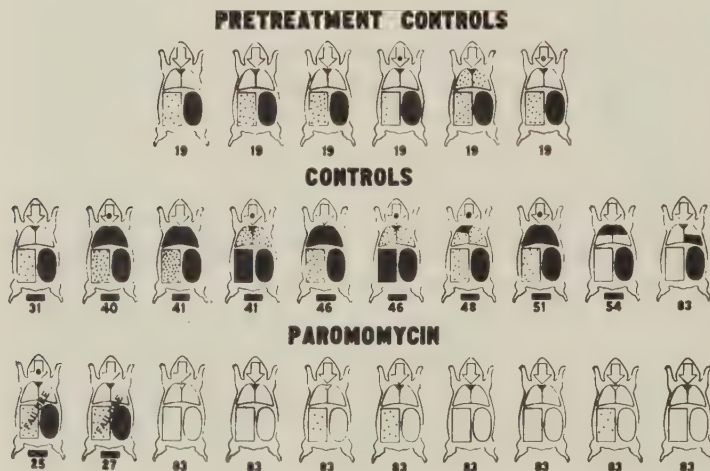


FIG. 3. Activity of paromomycin against experimental tuberculosis in guinea pigs, performed by Dr. A. G. Karlson, Mayo Clinic.

teria, and species of *Shigella*. Paromomycin was comparatively ineffective as a bacteriostat against *Ps. aeruginosa*, an observation that was confirmed in the bactericidal tests to be described.

Bactericidal Activity of Paromomycin Against Klebsiella pneumoniae In Vitro. As shown in figure 1, paromomycin at concentrations of 3.3, 6.7, 33.3, and 66.7 µg./ml. was clearly bactericidal in that sterilization occurred within 120 minutes at the lowest concentration and within 15 minutes at the highest concentration. The rate of killing action was roughly proportional to the amount of paromomycin present. It should be noted that this bactericidal activity was evident at 3.3 µg. paromomycin/ml., a concentration only slightly greater than the minimal 100 per cent inhibitory concentration of 1.6 µg./ml. shown in table I. Moreover, this bactericidal effect was obtained against a rather heavy bacterial suspension of 5×10^6 cells/ml.

Bactericidal Activity of Paromomycin Against a Heavy Suspension of Various Bacteria In Vitro. The results presented in table II reveal that 500 µg. paromomycin /ml. effected a marked bactericidal action within 1.5 hours against *Str. pyogenes*, *Staph. aureus*, *E. coli*, and *Proteus* species. With the exception of *E. coli*, the rate of bactericidal activity decreased with lesser concentrations of paromomycin. *Ps. aeruginosa*, however, proved to be insusceptible to killing by paromomycin within the seven hour observation period.

Bactericidal Activity of Paromomycin Against Fecal Flora of Mice. When the drug, at a concentration of 1.0 mg./ml., was added to a heavy suspension of mouse feces in vitro, it was found that complete eradication of the gram-negative aerobic flora occurred within 90 minutes. It was also observed that there was a marked reduction in the number of cultivable aerobic gram-positive bacteria within 90 minutes; however, a small number of microorganisms remained viable throughout the six and one half hour test period. These persisters proved to be species of *Staphylococcus* and *Candida*. Since these surviving staphylococci were subsequently found to be paromomycin-sensitive by broth-dilution test, it is difficult to account for the failure of paromomycin to eradicate completely these bacteria.

Activity of Paromomycin Against Experimental Bacterial Infections of Mice. The data in table III provide a spectrum of paromomycin activity against bacteria in vivo, with most of the infections being of an acute systemic nature. In general, the degree of paromomycin activity in vivo paralleled its inhibitory activity in vitro shown in table I. When administered subcutaneously as a single dose, the drug exhibited good to marked activity against strains of *Staph. aureus*, *K. pneumoniae*, *E. coli*, *Para. arizonae*, *P. mirabilis*, *P. vulgaris*, *Sh. flexneri*, and *Sh. sonnei*. Paromomycin was much less effective against the *Ps. aeruginosa* infections. Although 150 mg./Kg./day was required to effect a 50 per cent protection against a *Sal. typhimurium* infection, all surviving mice proved to be free of *Salmonella* by negative culture results from specimens of blood, liver, and spleen. It was also found that paromomycin given subcutaneously was about 20 to 50 times more effective than when given perorally. Moreover, when based on the 50 per cent effective dose level, a single subcutaneous dose of paromomycin was equal to, and in one instance better than, multiple subcutaneous doses.

Bactericidal Action of Paromomycin Against Klebsiella pneumoniae In Vivo. The effect of paromomycin at various dose levels (given as a single subcutaneous

injection) against an established, severe *Klebsiella* septicemia in mice is depicted in figure 2. Whereas dosages of 2.5 and 5.0 mg./Kg. caused a transient and limited reduction in the number of bacteria in the blood, the higher doses of 25 and 50 mg./Kg. rapidly reduced the degree of septicemia and effected a cure. Under these conditions, the 50 per cent curative dose of paromomycin would fall between 5 and 25 mg./Kg.

Activity of Paromomycin in Experimental Murine Tuberculosis. The results from three laboratories, given in table IV, indicate that paromomycin administered subcutaneously effected a definite suppressive action against *Myco. tuberculosis* in mice. Generally, the minimal effective dose was between 50 and 100 mg./Kg./day, and at comparable doses paromomycin was somewhat less active than streptomycin. Although some evidence was obtained, as shown in the upper part of table IV, that two divided doses of paromomycin were more effective than single daily doses, this was not substantiated in subsequent experiments.

Activity of Paromomycin in Experimental Guinea Pig Tuberculosis. The experiments performed at the Trudeau and Mayo Clinic laboratories indicated that paromomycin had a distinct antituberculosis effect against established disease in guinea pigs. The results of the Trudeau experiment, presented in table V, showed that paromomycin caused a marked prolongation of the survival time at subcutaneous dosages of 50 and 100 mg./Kg./day. This antibiotic was less active than streptomycin in terms of survival time and in degree of gross pathology. Again, as noted previously in the mouse tuberculosis experiments, single daily doses were about as effective as two divided doses per day.

Somewhat better activity was observed in the Mayo Clinic experiments than was found at the Trudeau Laboratory, as shown by the results symbolized in figure 3. A possible explanation of greater activity obtained at the Mayo Clinic laboratory is that the animals were treated longer (64 days as compared to 48 days at the Trudeau Laboratory) and they were sacrificed immediately after treatment was concluded (the guinea pigs in the Trudeau experiment were sacrificed 42 days after the last paromomycin dose).

DISCUSSION

The studies reported herein reveal that paromomycin is a highly effective antibacterial antibiotic, with impressively broad bacteriostatic and bactericidal activity both in vitro and in vivo. This antibiotic appeared to be especially active against staphylococci and gram-negative enteric bacteria. Paromomycin was highly bactericidal in test systems involving a strain of *K. pneumoniae*, in that 3.3 µg./ml. sterilized a heavy suspension of this organism within two hours in vitro. Similar bactericidal activity was suggested by an experiment with this strain in vivo following a single subcutaneous dose of 25 mg./Kg. in septicemic mice. Although the immune mechanisms of the mice doubtless contributed to the complete eradication of the *Klebsiella*, preliminary data from other experiments in immunologically depressed mice indicate that paromomycin by itself was truly bactericidal in vivo.

The greater activity of paromomycin given parenterally than when given perorally is most likely due to its poor absorption from the intestinal tract, a phenomenon that has been reported previously.¹ The retention of paromomycin in the intestines,

however, and its activity against fecal microflora suggest that this antibiotic may be useful for the treatment of bacterial enteridites and for the elimination of various bacteria from the gut prior to bowel surgery. In this respect, the persistence of staphylococci in feces in vitro, despite the presence of a high concentration of paromomycin, merits further scrutiny, particularly with regard to the question of whether such persistence occurs in vivo.

Since the parenteral administration of paromomycin is accompanied by certain intolerances¹ the use of high doses or prolonged treatment by this route would impose some limitations on the applicability of paromomycin for systemic bacterial infections. This consideration, in conjunction with the generally moderate activity of this antibiotic in experimental tuberculosis infections, appears to preclude the extensive parenteral use of paromomycin in tuberculosis. Nevertheless, the pronounced bactericidal activity, which has been demonstrated in these studies, would recommend this antibiotic for topical antibacterial therapy.

SUMMARY

A series of experiments were performed to delineate the behavior of paromomycin against medically important bacteria in vitro and in vivo. This antibiotic was shown to be bacteriostatic, at less than 10 µg./ml., against a wide variety of bacteria in vitro. Paromomycin was especially effective against staphylococci and gram-negative bacilli as an inhibitor in vitro and in experimental infections in mice. This antibiotic also exhibited marked bactericidal activity in vitro and in vivo under severe test conditions. Paromomycin given parenterally was moderately tuberculostatic in mice and guinea pigs. The therapeutic implications of these observations are discussed.

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Paromomycin as a Therapeutic Substance for Intestinal Amebiasis and Bacterial Enteritis

KENNETH O. COURTNEY, PAUL E. THOMPSON, ROBERT HODGKINSON,
AND J. R. FITZSIMMONS

Research Division, Parke, Davis & Co., Ann Arbor, Mich.

Paromomycin* is an antibiotic obtained from a culture of *Streptomyces* origin.¹ After laboratory research on its microbiological and pharmacological properties,^{2, 3, 13} a long program of clinical evaluation was initiated; many facets of the latter work still are in progress. The purpose of this report is to give a concise summary of the important features of paromomycin observed in the laboratory and to indicate the present status of the drug relative to the chemotherapy of certain of the enteric diseases.

LABORATORY STUDIES

Microbiological Properties in Vitro and in Experimental Animals. Paromomycin is highly active in vitro against many types of both gram-negative and gram-positive bacteria.^{2, 11, 13} It also proved to be very effective when tested parenterally against systemic bacterial infections in mice.^{1, 4, 13}

This antibiotic also has very high activity against *Entamoeba histolytica*.³ It was about twice as potent as emetine in five parallel tests against the UC strain. Moreover, the antiamebic effects of paromomycin were sustained when the supporting bacteria had been rendered resistant to the drug.³ It has strong antiamebic effect at 2 γ /ml. but only slight antibacterial effect at 70 γ /ml., which leaves little doubt that the drug has direct amebicidal effect in addition to indirect effect through the inhibition of normal bacteria.

Even more important, the drug is effective against amebae in vivo. When fed in the diet the antibiotic was effective in experimentally infected rats.³ Even smaller dosages are effective by gavage in rats.³

Severe amebic dysentery in dogs responded promptly to oral dosages of only a few mg./Kg./day and usually were cured after 10 days of such treatment.³

The development of amebic abscess of the liver in golden hamsters also was inhibited by treatment with paromomycin.³ Owing to very poor absorption when administered orally, even large doses were only moderately suppressive. Parenteral doses were more effective. Inasmuch as the drug persists in the plasma of animals for only a few hours after parenteral injection,² it is most reasonable to expect that many daily doses would be more effective than only one or two daily doses. This expectation was borne out by the observation in hamsters of significant effect by lower dosages when given in six daily portions than in two daily portions.

It is also noteworthy that the drug has shown chemotherapeutic effect in animals against several parasites other than *E. histolytica*. Time does not permit reviewing

* The trade name of Parke, Davis & Co. for paromomycin is Humatin.

the detailed observations along this line except to report that effects can be shown against all of the intestinal amebae in Rhesus monkeys, intestinal trichomonads in dogs, *Trichomonas foetus* and *Trichomonas vaginalis* in vitro and in mice, and against pinworms in mice.

Toxicity. Paromomycin is hardly absorbed at all from the gastrointestinal tract and it is very nontoxic when given orally. Mice, rats, monkeys, and dogs all tolerated very large amounts of the drug, when given in either one or many doses.²

Man also has been found to tolerate well several grams of the drug daily by mouth. The only definite side effect has been an increase in the number of stools, but this is not accompanied by nausea or intestinal cramping.

Animal studies showed that the parenteral administration of large dosages of paromomycin leads to kidney damage.² This effect is reversible and proportional to the dosage.

It remains to be determined whether the parenteral administration of paromomycin to man will cause kidney damage, but it is to be emphasized that oral use of the drug in many hundreds of patients has not resulted in either clinical or laboratory evidence of nephrotoxicity.

CLINICAL TRIALS IN INTESTINAL AMEBIASIS

After the extensive studies of paromomycin as an amebicidal agent just outlined, it was concluded from the results obtained in vitro and in experimental animals that paromomycin would probably be an unusually effective agent in the treatment of human intestinal amebiasis.

The validity of the conclusions drawn from these experimental observations has been more than amply borne out by the results reported from various parts of the world concerning extensive trials in human intestinal amebiasis. More than 400 reports on cases of intestinal amebiasis treated with paromomycin have been received from 13 investigators working in 12 different countries.

Type of Patients. Among these patients, the clinical form of the disease ranged from chronic cyst-passing, through the moderately acute to the very acute type of case. Furthermore, the cases reported varied from the moderately well nourished and well nourished in countries of a high socioeconomic level, to the anemic, poorly nourished patients in countries of a very low socioeconomic level.

Therapy. With the view to determining the minimum effective dosage required, the dosage of the drug was varied from 5 to 66 mg./Kg./day. Generally, the number of days constituting a course of treatment was five. Two groups, one of 20 and one of 10 patients, received 10 days of therapy; one group of 10 patients received 14 days' therapy; and two groups of 10 patients each were given the drug for only three days.

Side Effects. Three investigators reported that beginning on the second or third day of therapy the number of daily stools was increased by two or three movements. These were soft to loose, but never watery, in consistency. There was no abdominal cramping, nausea, or vomiting, and the bowel pattern returned to normal immediately on completion of therapy. Only one patient of the 432 reported had nausea and dizziness; these reactions disappeared when therapy was suspended. The other investigators consistently reported no adverse gastrointestinal effects and no other side reactions from the use of paromomycin.

Summary of Investigators' Reports (Table I). Paromomycin was used by Carter⁶ in the treatment of 104 patients with chronic and subacute intestinal amebiasis at the Sunland Training Center in Florida. These patients ranged from 2 to 30 years of age and were institutionalized due to epilepsy, mental retardation, or spasticity. They were treated with dosages ranging from 5 to 66 mg./Kg./day for 1 to 5 days. Results were assessed within a few days after the end of treatment, and long follow-up was not attempted in the majority of cases because of the difficulty of distinguishing between relapse and reinfection. Of the 104 patients treated, 49 were treated with 12.5 mg./Kg. daily and all but 4 were cleared. These 4 were cleared after a second course of treatment. Of those patients treated with more than 12.5 mg./Kg./day, all were cleared. The 16 patients treated with 5 mg./Kg./day were not cured.

Paromomycin was used in the treatment of 22 patients with subacute and protracted acute intestinal amebiasis in Panama. Treatment was for a five day period, using 25 mg./Kg. of body weight per day. Symptomatic improvement was noted on the second and third days, and by the end of the fifth day of treatment, all patients were feeling well and the stools were negative for amebae. Follow-up of these cases

TABLE I
Paromomycin Trials in Intestinal Amebiasis (Given Orally, Usually 3 Daily Doses)

Geographic area	No. of patients	Type of infection	Treatment mg./Kg./day	Days	No. of patients examined and no. negative on days after treatment began				
					1-7 exam./neg.	8-14 exam./neg.	15-30 exam./neg.	31-59 exam./neg.	60 and over exam./neg.
Chile	15	Chronic	25	5			11/11		
Egypt	20	Acute and chronic	10-20	7-14	20/19	19/19	19/17	17-15	15/13
England	19	Chronic	30	10	18/16	18/16	1/1	6/5	4/4
Ethiopia	97	Acute and chronic	7.5-30	2-5	79/79	70/70	35/32	36/36	15/15
Honduras	8	Acute and chronic	22-28	3-4	← * →				
Mexico	26	Acute and chronic	18-25	5	2/2	26/23	26/23		
Nicaragua	41	Acute and chronic	12.5-25	5	4/4	31/31	1/1	← † →	
Panama	22	Acute and chronic	25	5	← ‡ →				
Philippines	18	Acute and chronic	30	3-5	14/13	5/5	4/4	1/1	
Puerto Rico	20	Acute and chronic	4-26	5	20/19	20/19	14/14	10/10	10/10
United States	32	Acute and chronic	10-20	5	29/26	28/28	31/30	25/25	30/29
United States	104	Acute and chronic	5-66	1-5	36/29	31/23	60/40	19/12	21/14
Union of South Africa	10	Acute	2.4 Gm./day	10			10/6		
Totals	432				222/207	248/234	212/179	114/104	95/85

* Time interval for stool examinations not recorded. However, investigator reported all cases "cured."
† Time interval for stool examinations not recorded for 5 patients. However, investigator reported all cases "negative."
‡ Time interval for stool examinations not recorded. However, investigator reported all cases "negative."

for from 10 to 30 days after the completion of treatment revealed that the patients were still negative for amebae.

A study in Puerto Rico⁷ of 20 cases of chronic and subacute intestinal amebiasis was made using dosages ranging from 4 to 26 mg./Kg./day. The duration of treatment was five days. These patients were followed for a 90 day period at the end of which all remained free of amebae.

The antibiotic was studied in Florida in 32 children with acute and chronic intestinal amebiasis. Dosages varied from 10 to 20 mg./Kg./day for a five day period. An 11 week follow-up, with stool examinations at the first, second, third, fourth, sixth, and eleventh weeks, revealed at the end of this period only two recurrences among the 32 patients.

In Honduras, a series of 8 patients with acute intestinal amebiasis were treated with the drug in dosages of 22 to 28 mg./Kg./day for a three to four day period. Five of these patients were completely cured; 2 required a second course of treatment. One patient, who had a three year history of acute amebiasis and had previously been treated with virtually all known forms of therapy, required a third course of treatment, but this course was effective and the patient has remained negative for more than two months.

Forty-one patients with subacute to fulminating acute intestinal amebiasis were treated in Nicaragua. Treatment was for a five day period; 16 patients received 12.5 mg./Kg./day, 20 patients received 25 mg./Kg./day, and 5 received 18 mg./Kg./day. In all cases, symptomatic relief was obtained on the third or fourth day, the stools were negative on the fourth and fifth days, and a subsequent follow-up of periods varying from 10 to 21 days revealed that all except 1 of the 41 patients remained negative for amebae. This one case cleared with a second course of treatment.

Shafei,⁸ in Egypt, after a 90 day follow-up on a series of 20 cases for which he used dosages of 20 mg./Kg./day for seven days in 10 cases and 10 mg./Kg./day for 14 days in the remaining 10 cases, reported a 70 per cent cure rate.

A six month post-therapy follow-up in 19 cases of chronic intestinal amebiasis in England, after a dosage of 30 mg./Kg./day for a 10 day period, resulted in an estimated 95 per cent cure rate.

In Ethiopia, Wagner⁹ treated 97 cases of intestinal amebiasis of the moderately to severely acute form of the disease, with dosages varying from 7.5 to 30 mg./Kg./day, for two to five days. Follow-up examinations were made (depending on the cooperation of the patients) for a minimum of 18 days in some cases to a maximum of 110 days in others. Of the 97 patients treated, all but 3 were clinically and parasitologically cured. Of these 3, 2 cleared parasitologically with a second five day course of treatment.

In South Africa, 10 patients, all having ulceration of the bowel, were treated with 2.4 Gm. daily for 10 days. Sixty per cent appeared to be cured during a 27 day follow-up.

Fifteen patients with amebic dysentery were treated with 21 mg./Kg./day for five days by Dooner,¹⁰ in Chile, and of the 11 who returned for follow-up, one to three months after treatment, all were clinically and parasitologically cured.

Twenty-six patients were treated in Mexico with 18 or 25 mg./Kg./day for five days. Of the 24 nondysenteric and 2 dysenteric amebic patients returning for follow-

up examinations 15 to 30 days after therapy, 23 were symptomatically and parasitologically negative.

In the Philippines, 18 patients with acute intestinal amebiasis were treated with 25 mg./Kg./day for three or five days. Of the 17 patients who returned for follow-up one to three weeks after therapy, 1 required a second course of treatment while all others remained well and parasitologically negative.

It is noteworthy that all investigators uniformly reported marked relief in all symptomatic patients beginning as early as the second or third day of treatment.

In summarizing the results of these studies, it would appear that paromomycin, in dosages of 15 to 25 mg./Kg. of body weight per day for five days, is eminently suited for the treatment of most cases of chronic and acute intestinal amebiasis.

THE TREATMENT OF ENTERIC BACTERIAL INFECTIONS

The results of antibacterial inhibition tests in vitro and in experimental infections in mice have indicated that paromomycin has significant activity against groups of bacteria associated with enteric infections in man. These bacteria were most notably species of *Salmonella*, *Shigella*, *Paracolobactrum*, and enteropathogenic strains of *Escherichia coli*.¹³ Although definitive clinical trials are still in progress, the present results have shown that orally administered paromomycin was effective in controlling human enteritis caused by the afore-mentioned bacteria. Some representative examples are described in the following section.

Shigellosis and Infantile Diarrhea. An explosive outbreak of enteritis due to *Shigella flexneri* occurred among 160 inmates of a midwestern children's institution. Approximately half of these patients were given oral paromomycin at a dose of 25 mg./Kg./day, and half were given 50 mg./Kg./day, with both groups treated for six to seven days. Only 4 patients from each group failed to respond promptly, and stools from only 2 of these 8 patients yielded *Shigella* on culture.⁵

In another study, bacteriological and clinical clearing of shigellosis occurred among 6 of 7 children given oral paromomycin at 50 mg./Kg./day for seven to nine days. An additional 17 children, in the same study, were treated with the same regimen of paromomycin for infantile diarrhea caused by various enteropathogenic serotypes of *E. coli*. Fifteen of these 17 patients showed a prompt bacteriological and clinical response; the 2 patients who did not respond, even after 14 days of paromomycin treatment, were infected with the *E. coli* 0127 serotype.¹²

Salmonellosis. Paromomycin was employed for the treatment of *Salmonella typhimurium* infection among a family of 5 in Detroit after two and one half months of treatment with other drugs had failed to eradicate these bacteria from the stools. Four of these 5 patients became bacteriologically negative after five day oral courses of 25 mg./Kg./day, and the other patient became negative after a second five day course at the same dosage. All 5 patients remained well and their stools continued to be free of *Salmonella* throughout a five month follow-up period.⁵

An additional 38 patients with enteritis due to *Sal. typhimurium* in a London hospital were treated by McMath et al¹¹ with oral paromomycin doses of 25 to 75 mg./Kg./day for five days. Most of these patients had not responded to treatment with other agents. Of these 38, 31 patients underwent a bacteriological conversion subsequent to paromomycin treatment.

Paromomycin was also used by Ross¹² for the treatment of salmonellosis in 9 children, 7 of whom no longer had pathogens in their stools after seven to nine day oral courses of 50 mg./Kg./day. Ross also reported no side effects and no abnormalities in serial hemograms, urinalyses, or blood urea nitrogen and thymol turbidity tests among his paromomycin-treated patients.

SUMMARY

Paromomycin is unique among known antibiotics to the extent that it is characterized by direct and marked amebicidal action and high activity against a wide range of enteric bacteria. It is well tolerated due to almost a total lack of absorption following oral administration. In view of this array of properties, as well as the excellent results obtained in more than 1000 patients, it would appear that paromomycin will be especially useful for the treatment of various enteric infections.

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Paromomycin in Diarrheas of Infants and Children

G. D. GODENNE

*Department of Pediatrics, The Johns Hopkins University
School of Medicine, Baltimore, Md.*

The purpose of this study was to investigate the clinical and bacteriological effectiveness of paromomycin* in diarrheas of infants and children. Paromomycin has been shown to be an antibiotic with a broad antibacterial spectrum similar to that of neomycin and with limited absorption by the gastrointestinal tract.

CASE MATERIAL AND METHOD OF STUDY

The infants and children reported in this study were patients of the Harriet Lane Home of the Johns Hopkins Hospital and of the Pediatric Service of the Baltimore City Hospital.

Fifty-five patients were given paromomycin, 31 were in-patients and 19 were out-patients of the Harriet Lane Home, and 5 children were hospitalized at Baltimore City Hospital.

All of the presumably infectious diarrheas were treated with paromomycin over a period of two months. A rectal swab was taken on the first day of treatment and on the fourth day. The swab was streaked on desoxycholate agar, desoxycholate citrate agar, and then was placed in a tube containing selenite-F enrichment medium. The plates and the broth were cultured in the routine fashion in the laboratories of the Johns Hopkins Hospital.

Clinical progress was ascertained from the clinical observation by the resident staff. Signs of intolerance were carefully looked for.

Paromomycin was at first given at a dose of 50 mg./Kg./day; this dose was soon increased to 100 mg./Kg./day as the patients failed to show any response on the small dosage. Paromomycin was given in four divided doses daily and was discontinued after six days of treatment.

At the time of this study, paromomycin was an experimental drug and was available in capsules containing 150 mg. Small infants were given the content of a capsule in a syrup vehicle.

RESULTS

Fifty-five children with moderately severe to severe diarrhea were given paromomycin; 50 children were given paromomycin to the exclusion of all other drugs, and 5 children were given paromomycin in combination with chloramphenicol.

The patients are divided in four groups (table I). Group 1 includes 17 infants and children who grew *Salmonella* or *Shigella* in their stools. These children ranged from 2 months to 7 years of age, 9 were less than 1 year, 3 were between 1 and 4 years, and 5 children were more than 4 years old.

* The trade name of Parke, Davis & Co. for paromomycin is Humatin.

TABLE I
Age Groups of Children Studied

Group	Total	Number of infants and children given paromomycin			
		Premature infants	Less than 1 year	Between 2 and 4 years	More than 4 years
1	17	0	9	3	5
2	19	1	13	2	3
3	14	9	4	0	1
4	5	2	2	1	0

Group 2 includes 19 children ranging from 1 day to 6 years of age who showed no pathogenic organisms in their stools. In this group 13 children were between 1 day and 1 year, 2 were between 1 and 4 years, 3 between 4 and 6 years, and 1 was a premature weighing 1680 Gm. The organisms isolated in the stools of this group were: *Proteus*, paracolon, *Klebsiella*, *E. coli*, and *Pseudomonas*.

Group 3 includes 14 children with incomplete laboratory data. They all had a stool culture on the first day of treatment, which showed similar organisms to those mentioned in group 2, but for technical reasons the culture was not repeated on the fourth day. This group includes 9 premature babies whose weights varied between 1330 and 2175 Gm., 4 children less than 6 months, and a 7 year old child.

Group 4 represents 5 children who were treated with a combination of chloramphenicol and paromomycin. Two were premature infants, 2 were less than 1 year, and 1 was 2 years old. The results of treatment of these four groups are shown in table II. In the group of diarrheas due to *Salmonella* or *Shigella*, 76 per cent improved clinically after four days of treatment and 47 per cent had negative cultures for *Salmonella* or *Shigella* after four days. In the groups of diarrheas in which no pathogenic organisms were seen on the stool culture, 90 per cent improved clinically and 100 per cent showed changes in stool culture, 30 per cent of which were sterile after four days. In the 5 cases of diarrhea treated with both drugs, the clinical and bacteriological changes did not seem to be superior to those occurring in cases treated only with paromomycin. In 4 cases yeastlike organisms appeared in the stool culture after four days of treatment.

TABLE II
Results of Treatment

Group	No. patients	Clinical changes after 4 days		Bacteriological changes		
		None	Improved	Suppression of <i>Salmonella</i> or <i>Shigella</i>	Changes in flora	Sterile
1	17	4	13	8	17	0
2	19	0	19	—	13	6
3	14	3	11			
4	5	3	2			

DISCUSSION

It appears from these data that infectious diarrheas due to *Salmonella* or *Shigella* respond favorably from a clinical standpoint in 76 per cent of the cases treated with paromomycin. A favorable clinical response was based on the following criteria: changes in the appearance of the stools, diminution of their frequency, and absence of blood in the stools.

In almost 50 per cent of the cases the pathogenic organism was absent from the stool after four days. Longer bacteriological observation was not possible in order to ascertain the relapse rate.

In 100 per cent of the patients given paromomycin the bowel flora showed some changes and in 30 per cent the bowel was sterile after four days.

The interpretation of the 90 per cent rate of improvement in groups 2 and 3 is difficult, since many cases of diarrhea will improve without specific antibacterial medication. Premature infants and children did not show any sign of intolerance to the administration of paromomycin. At no time were toxic rashes noted; there was no recurrence of vomiting during the administration of the drug and the urines remained normal after the course of treatment.

SUMMARY

Paromomycin alone and in combination with chloramphenicol was evaluated in the treatment of a group of premature infants and children with diarrhea. All patients given paromomycin had changes in their bowel flora after four days, 30 per cent of whom had sterile cultures after four days. Among the 17 patients treated for *Salmonella* or *Shigella* infections, 47 per cent did not grow the organism after four days. In all patients the drug failed to show any toxic effects. No particular advantage over other accepted antibiotics was observed.

The Action of Certain Phthalic Acid Derivatives upon Poliovirus Growth in Tissue Culture

ARIEL C. HOLLINSHEAD AND PAUL K. SMITH

*Department of Pharmacology, The George Washington University
School of Medicine, Washington, D. C.*

More than 100 phthalic acid derivatives have been studied for their inhibitory effect against poliovirus in tissue cultures of HeLa cells or of monkey kidney. In these systems the media regularly contained serum so it became of interest to learn whether the toxic and chemotherapeutic activities of the drugs would be the same in a nonprotein medium infected with purified poliovirus. Accordingly, a number of these drugs were tested against poliovirus in the same cell systems without added serum.

MATERIALS AND METHODS

Tissue Cultures. Monkey kidney and HeLa cell cultures were grown out in culture media described by Melnick et al.,⁸ Eagle,¹⁻⁴ and Eagle and Habel⁵ respectively. Cultures were thoroughly washed, maintained in serum-free media overnight, washed again and maintained in serum-free media for all virus growth experiments.

Virus. Plaque-purified type 1 Mahoney strain poliovirus passed and titered in each type of cell culture was used throughout the study. Purification of the virus was done by a process involving Spinco ultracentrifugation and the use of cellulose anion exchange columns.⁷

Drugs. The drugs were prepared for use and tested as described by Hollinshead and Smith.⁶ The first six compounds listed in table I were chosen for a study in which cell protein left on the cellulose anionic exchange columns after elution of virus nucleoprotein was eluted, extracted and added with the drug to the purified virus to see whether addition of this cell protein might affect cell binding or drug toxicity in any way. In another set of experiments the cell protein was added with the virus to see whether it might affect cell entry of the virus before addition of drug or cell binding of the virus in the presence of drug. Monkey kidney cell cultures were used for these experiments.

In order to study a possible antimetabolite effect of one of the inhibitors, each of the 14 amino acids contained in Rappaport's SM-2 media were increased in separate experiments in graded amounts from 1 to 0.008 mg./ml. in the presence of 1-(2H)-phthalazone, 2-(*p*-aminophenyl)-4-methyl at twice its minimum inhibitory concentration.

RESULTS AND DISCUSSION

The results of these studies are presented in table I. It may be seen that those compounds that were active against the virus usually exhibited the same or a nar-

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TABLE I

*Effect upon Poliovirus Infections of Tissue Cultures by Certain
Phthalic Acid Derivatives*

Derivative	Therapeutic indices at 72 hours, mg./ml.			
	Serum plus crude virus		No serum plus pure virus	
	Kidney	HeLa	Kidney	HeLa
1-(2H)-Phthalazone, 2-(<i>p</i> -aminophenyl)-4-methyl	1.0/0.25*	1.0/0.06*	1.0/0.25	1.0/0.12
Phthalide, 3,3'-bis-(2,4,6-trihydroxyphenyl)-	0.12/0.12*	0.25/0.06*	0.12/0.12	0.5/—
Phthalimide, N-methyl	0.25/0.015	2.0/0.12	0.5/0.25	1.0/0.5
Phthalimide, N-2-hydroxyethyl	2.0/0.03	2.0/1.0	2.0/0.5	2.0/2.0
Phthalimide, N-isobutyl	0.12/0.03	2.0/0.015	0.5/0.12	2.0/0.06
Phthalimide, N-propyl	0.5/0.12	1.0/0.06	0.25/0.12	1.0/—
Phthalimide, N-isopropyl	0.5/0.5	2.0/0.5	0.25/—	1.0/—
Phthalimide, N-1-naphthyl	4.0/2.0	0.25/0.25	1.0/1.0	1.0/—
Phthalimide, N-(9-oxo-2-fluorenyl)-	0.004/0.004	0.015/—	0.004/—	0.008/—
Phthalimide, N-(<i>p</i> -tolyl)-	0.03/—	0.03/—	0.03/—	0.015/—
Phthalimide salt, K	2.0/1.0	1.0/0.03	2.0/—	0.5/—
Phthalide, 3,3'-bis-(2,4,6-trihydroxy- <i>m</i> -tolyl)-	0.5/0.25	0.25/0.12*	0.12/0.12	0.25/—
Phthalyl-DL-valine	0.12/0.06	0.12/—	0.06/0.06	0.12/—
Phthalimide, N-(1-methylbutyl)-	0.3/—	0.3/—	0.015/—	0.3/—
Phthalic Acid, 3-hydroxy	0.25/0.12	1.0/—	0.25/0.25	1.0/—
Phthalic Acid, 3-nitro	0.06/0.06	0.06/—	0.06/—	0.03/—
Phthalic Acid, 3,4,5,6 tetrachloro	0.06/0.06	0.03/—*	0.06/—	0.03/—
Phthalic Acid, diester with tetrahydro-2-furanpropanol	0.12/0.12	1.0/—*	0.12/—	0.12/—

*The potassium phthalimide salt was obtained from Brothers Chemical Co., the phthalyl-DL-valine was obtained from H. M. Chemical Co., and the rest of the compounds were obtained from Chemical Biological Coordination Center. Data obtained by Dr. Glenn Fischer.

rower range of virus inhibition in the serum-free synthetic systems infected by the purified virus nucleoprotein, outstanding examples being N-methyl phthalimide and N-2-hydroxyethyl phthalimide in the kidney system and N-methyl phthalimide and N-isobutyl phthalimide in the HeLa system. Those compounds, which were borderline inhibitors in the serum-containing system, were usually inactive in the system without serum. Usually the compounds were less toxic in the serum-fed systems. The inhibitors were all virustatic except for phthalyl-DL-valine which was virucidal in its narrow inhibitory range.

It was found that five of the six compounds were nonspecific in their effect against virus or cell nucleoproteins in that the effects of drug or virus were not reduced in the presence of cell protein. Virus inhibition by 1-(2H)-phthalazone,2-(*p*-aminophenyl)-4-methyl was reduced to a ratio of one in the presence of added cell protein tested in graded amounts from 10 to 1 mg. Less than 4.5 mg. failed to have any effect on the phthalazone compound. The presence of serum in the media prevented this effect from occurring.

Except for the known inhibitory effect of glycine upon poliovirus and the growth-promoting effect of cysteine, which were paralleled in the controls, there were no effects seen as a reversal of the inhibitory effects of the phthalazone by any of the amino acids added.

SUMMARY

Purified poliovirus type 1 strain Mahoney was grown in two separate tissue culture systems each fed with definable serum-free synthetic media. The effects of certain phthalic acid derivatives upon the growth of the virus were compared with the results obtained in the same system but using serum-containing media. Those compounds which were active usually exhibited a narrower range of virus inhibition in the serum-free systems and some compounds were more toxic to the host cells. All of the compounds except 1-(2H)-phthalazone, 2-(*p*-aminophenyl)-4-methyl were nonspecific in their effects against virus or cell nucleoproteins in that the effects of the drugs were not reduced in the presence of cell-protein. Virus inhibition by the phthalazone was reduced in the presence of added cell protein and the reduction was prevented by the presence of serum. None of 14 amino acids reversed inhibition of poliovirus by the phthalazone.

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Studies on Synergists for Antimicrobial Agents

III. Compounds Having Plant Growth Activity

GEORGE H. SCHERR* AND ROBERT M. BECHTLE

*Department of Microbiology, The University of Illinois
College of Medicine, Chicago, Ill.*

Previous studies with the strip-gradient plate technique had shown that compounds such as α -(2,4,5-trichlorophenoxy) propionic acid and 2,4,6-trichlorophenoxyacetic acid demonstrated excellent in vitro potentiating (synergistic) activity for antibiotics against microorganisms. Consequently a series of related compounds were investigated using materials and methods previously described.^{1,2}

MATERIALS AND METHODS

Strip-gradient plates were used to test the potentiating activity of 24 agents against *Sarcina lutea*, *Staphylococcus aureus* 209, and *Mycobacterium tuberculosis* 607, using the antimicrobial agents penicillin, chloramphenicol, erythromycin, tetracycline, chlortetracycline, oxytetracycline, ristocetin, polymyxin B, bacitracin, isoniazid, *p*-aminosalicylic acid, pyridine-3-sulfonic acid, and streptomycin.

Tables I, II, and III present the data for the organisms *S. lutea*, *Staph. aureus*, and *Myco. tuberculosis* 607, respectively. Table IV summarizes these data to show the relative differences among the different antibiotics acting with the 24 agents tested here for synergistic activity.

Figure 1 indicates how the dimensions "h" and "v" are used in these tables to quantitate the degree of synergism or antagonism as a factor (h/v).

RESULTS AND DISCUSSION

The agents tested as potentiators in this study proved to be the most active and versatile of any group of compounds studied to date. For example, 2,4,6-trichlorophenol, 2,4,6-trichlorophenoxyacetic acid, and α -(2,4,5-trichlorophenoxy) propionic acid showed marked synergistic activity with 11, 9, and 7 of the 13 antimicrobial agents, respectively. Also 2,4,6-trichlorophenol showed synergism with chloramphenicol against *S. lutea*, with erythromycin, tetracycline, chlortetracycline, oxytetracycline, ristocetin, and bacitracin against *Staph. aureus*, and with isoniazid, *p*-aminosalicylic acid, pyridine-3-sulfonic acid, and streptomycin against *Myco. tuberculosis* 607.

Included in this study were the compounds indole-3-propionic acid, alpha-naphthalene acetic acid, and indole-3-acetic acid because, although molecularly dissimilar, they share with many of the phenoxy acetic and phenoxy propionic acids the feature of having some aspects of plant growth regulating activity. These compounds did not display any activity that would either class them with or distinguish them from the other agents used in this work.

* Present address: Consolidated Laboratories, Inc., Chicago Heights, Ill.

TABLE I

Strip-Gradient Plate Results with Sarcina lutea

Agent on strip, 1000 µg./strip	Antibacterial agent in gradient*		
	None	Penicillin	Chloramphenicol
Mercaptoacetic acid	Tr Stim	Tr A	Neg
<i>o</i> -Toloxycetic acid	Neg	Tr S	Neg
4-Chloro- <i>o</i> -toloxycetic acid	Neg	S-30/5	Neg
<i>p</i> -Chlorophenoxyacetic acid	Neg	Tr S	Neg
2,4-Dichlorophenoxyacetic acid	Neg	Tr S	Neg
2,5-Dichlorophenoxyacetic acid	Neg	Tr A	Neg
3,4-Dichlorophenoxyacetic acid	Neg	S-20/10	Neg
2,4,5-Trichlorophenoxyacetic acid	Neg	Tr S	Neg
2,4,6-Trichlorophenoxyacetic acid	Neg	S-10/10	Neg
2,3,4,6-Tetrachlorophenoxyacetic acid	Neg	S-20/10	Tr S
Pentachlorophenoxyacetic acid	5 mm, Inh	S-20/15	S-5/65
beta-Mercaptopropionic acid	Tr Inh	S-6/10	Neg
alpha, alpha'-Dichloropropionic acid	Neg	Tr S	Neg
alpha-Phenoxypropionic acid	Neg	A-30/10	Neg
2(<i>p</i> -Chlorophenoxy) propionic acid	Neg	Neg	Neg
3(<i>p</i> -Chlorophenoxy) propionic acid	Neg	S-20/5	Neg
alpha-(2,4-Dichlorophenoxy) propionic acid	Neg	Tr S	Neg
2,4,5-Trichlorophenoxypropionic acid (alpha)	Neg	Tr S	Neg
3(2,4,5-Trichlorophenoxy) propionic acid	Neg	S-30/15	Neg
beta-Isothiouriedopropionic acid	Neg	Neg	Neg
Indole-3-propionic acid	Neg	Tr A	Tr S
alpha-Naphthaleneacetic acid	Neg	S-15/10	Neg
Indole-3-Acetic acid	Neg	S-5/5	Neg
2,4,6-Trichlorophenol	Tr Inh	Tr A & S	S-5/65

* Neg = no activity; Tr = trace of activity; Inh = inhibition of growth; Stim = stimulation of growth; S = synergism; A = antagonism.

TABLE II

Strip-Gradient Plate Results with Staphylococcus aureus 209

Agent on strip, 1000 µg./strip	Antibacterial agent in gradient									
	None	Penicillin	Chloramphenicol	Erythromycin	Tetracycline	Chlorotetracycline	Oxytetracycline	Ristocetin	Poly-myxin B	Bacitracin
Mercaptoacetic acid	Neg	Tr S	Tr S	Tr A	Tr S&A	Tr S	Tr A	A-10/10	Tr A	Tr A
<i>o</i> -Toloxycetic acid	Tr Stim	Neg	Neg	Neg	Neg	Neg	Tr A	S-15/10	Neg	Tr A
4-Chloro- <i>o</i> -toloxycetic acid	Neg	Neg	Neg	S-20/20	Tr A	Tr A	Tr A	Tr S	S-5/35	Tr S
<i>p</i> -Chlorophenoxyacetic acid	Neg	Neg	Neg	S-10/20	Neg	S-10/15	Neg	Neg	Neg	Tr S
2,4-Dichlorophenoxyacetic acid	Neg	Tr S	Neg	Tr S	Neg	Neg	Tr A	Neg	Neg	Neg
2,5-Dichlorophenoxyacetic acid	Neg	Neg	Neg	S-10/10	A-25/10	Tr A	Tr A	Neg	Neg	Neg
3,4-Dichlorophenoxyacetic acid	Tr Stim	Tr A	S-5/25	Tr S	Tr S	S-15/50	Tr A	S-15/15	S-5/35	S-10/10
2,4,5-Trichlorophenoxyacetic acid	Tr Stim	Tr A	S-15/20	S-10/5	Tr S	S-15/35	Tr A	A-20/5	S-5/60	A-10/10
2,4,6-Trichlorophenoxyacetic acid	Neg	S-10/25	S-12/5	S-25/30	Tr A&S	Tr A&S	Neg	S-25/15	S-20/35	S-22/10
2,3,4,6-Tetrachlorophenoxyacetic acid	3 mm Inh	S-5/35	A-5/30	S-20/10	Tr A	A-5/40	Tr A	S-5/40	A-5/30	A-15/20
Pentachlorophenoxyacetic acid	5 mm Inh	Neg	S-5/30	S-10/10	S-5/40	A-5/30	S-10/40	Neg	Neg	A-10/10
beta-Mercaptopropionic acid	Neg	S-5/10	Neg	A-40/15	A-5/5	S-10/25	Neg	Tr A	Tr A	S-10/15
alpha, alpha'-Dichloropropionic acid	Neg	Neg	Neg	A-15/15	S-5/10	S-30/30	Neg	S-30/10	S-7/20	S-12/20
alpha-Phenoxypropionic acid	Neg	Neg	Neg	S-20/10	Tr A&S	S-10/10	Neg	Tr A&S	Neg	Neg

Table II Continued on Page 318

TABLE II (Continued)

Strip-Gradient Plate Results with *Staphylococcus aureus* 209

Agent on strip, 1000 µg./strip	None	Antibacterial agent in gradient								
		Peni- cillin	Chlor- amphen- icol	Eryth- ro- mycin	Tetra- cycline	Chlor- tetra- cycline	Oxy- tetra- cycline	Risto- cetin	Poly- myxin B	Bacitra- cin
2(<i>p</i> -Chlorophenoxy) propionic acid	Neg	Neg	Neg	S-20/25	S-20/5	Neg	Neg	Neg	Tr S	Neg
3(<i>p</i> -Chlorophenoxy) propionic acid	Neg	Neg	Neg	S-15/10	A-10/5	S-10/5	Neg	S-25/10	Tr S	Tr S
alpha-(2,4-Dichlorophenoxy) propionic acid	Neg	Neg	Tr S	S-10/15	Neg	Tr S	Tr A&S	Tr S	S-5/35	Tr S
2,4,5-Trichlorophenoxy- propionic acid (alpha)	Tr Inh	S-12/20	Tr S	S-10/10	Tr S	Tr S	Tr S	S-25/15	S-10/60	S-10/20
3(2,4,5-Trichlorophenoxy) propionic acid	10 mm Inh	A-5/50	A-5/40	S-20/30	S-10/15	S-10/20	S-10/15	Neg	Neg	S-10/20
beta-Isothiouriedo- propionic acid	Neg	Neg	Neg	Neg	Tr S	Tr S	Tr S	Neg	Tr A	Tr S
Indole-3-propionic acid	Tr Inh	Neg	Tr S	Tr S	Tr S	Tr S	S-15/15	Tr A	Tr A	S-5/10
alpha-Naphthalene acetic acid	Neg	A-10/5	Neg	S-7/10	A-15/15	S-10/15	Neg	Tr A	Tr S	Neg
Indole-3-acetic acid	Neg	Neg	Neg	S-10/5	A-10/5	S-20/10	Neg	A-7/5	Neg	Tr A
2,4,6-Trichlorophenol	10 mm Inh	Neg	Tr S	S-15/45	S-25/30	S-25/30	S-30/35	S-20/35	Tr S	S-10/40

TABLE III

Strip-Gradient Plate Results with *Mycobacterium tuberculosis* 607

Agent on strip, 1000 µg./strip	Antibacterial agent in gradient				
	None	Isoniazid	<i>p</i> -Amino- salicylic acid	Pyridine- 3-sulfonic acid	Strepto- mycin
Mercaptoacetic acid	Neg	Tr A	Tr S	A-25/25	Tr A
<i>o</i> -Toloxycetic acid	Neg	Tr A	Neg	A-20/25	Tr A
4-Chloro- <i>o</i> -toloxycetic acid	Neg	Tr A	Neg	A-30/10	S-10/10
<i>p</i> -Chlorophenoxyacetic acid	Neg	Neg	Neg	Neg	Neg
2,4-Dichlorophenoxyacetic acid	Tr Inh	Neg	Neg	Tr A	Tr A
2,5-Dichlorophenoxyacetic acid	Tr Inh	Tr A	Neg	A-10/10	S-10/5
3,4-Dichlorophenoxyacetic acid	Neg	Tr A	Neg	A-15/10	Tr S
2,4,5-Trichlorophenoxyacetic acid	Tr Inh	S-10/10	Neg	A-10/5	Tr S
2,4,6-Trichlorophenoxyacetic acid	Neg	S-5/30	Tr S	Neg	S-10/5
2,3,4,6-Tetrachlorophenoxy- acetic acid	Tr Inh	Tr S	Tr S	Neg	Tr S
Pentachlorophenoxyacetic acid	10 mm Inh	A-5/25	A-5/40	A-5/40	S-10/10
beta-Mercaptopropionic acid	Neg	Tr A	Neg	A-30/50	Neg
alpha, alpha'-Dichloropropionic acid	Neg	Tr A	Neg	Tr S	Tr A
alpha-Phenoxypropionic acid	Neg	Tr S	Neg	Neg	Tr S
2(<i>p</i> -Chlorophenoxy) propionic acid	Neg	S-25/15	Neg	Neg	Tr S
3(<i>p</i> -Chlorophenoxy) propionic acid	Neg	Tr S	Neg	A-25/10	Tr A
alpha-(2,4-Dichlorophenoxy) propionic acid	Neg	S-10/20	Tr S	Tr A	Tr S
2,4,5-Trichlorophenoxy- propionic acid (alpha)	Neg	S-10/30	Tr S	Tr A	S-30/15
3(2,4,5-Trichlorophenoxy) propionic acid	10 mm Inh	A-5/5	Neg	S-5/15	Tr S
beta-Isothiouriedopropionic acid	Neg	Neg	Neg	A-25/50	Tr A
Indole-3-propionic acid	Neg	Tr A	Tr S	S-10/30	Tr S
alpha-Naphthalene acetic acid	Neg	Tr S & A	Neg	Tr A	Neg
Indole-3-acetic acid	Neg	Neg	Neg	Neg	Tr A
2,4,6-Trichlorophenol	10 mm Inh	S-30/25	S-5/40	S-5/50	S-15/10

TABLE IV

Number of Compounds out of 24 Tested which Showed Frank Synerism with Antibiotic*

Peni- cillin	Chlor- amphen- icol	Erythro- mycin	Tetra- cycline	Chlor- tetra- cycline	Oxy- tetra- cycline	Risto- cetin	Poly- myxin B	Bacitra- cin	Isoni- azid	p-Amino- salicylic acid	Pyridine- 3-sulfonic acid	Strepto- mycin
<i>Sarcina lutea</i> 10	2	—	—	—	—	—	—	—	—	—	—	—
<i>Staph. aureus</i> 209-P 4	4	16	5	11	4	8	7	8	—	—	—	—
<i>Myc. tuberculosis</i> 607 —	—	—	—	—	—	—	—	—	6	1	3	6

* Compilation of data from tables I, II, and III.

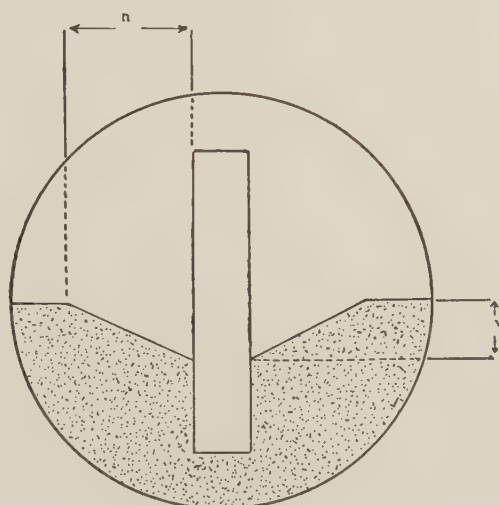
Pentachlorophenoxy acetic acid showed frank antagonism to 5 of the 13 drugs (tables II and III); there is no ready explanation for this characteristic.

Certain of the antimicrobial agents were particularly susceptible to potentiation by these agents. Thus, the antimicrobial activity of erythromycin was enhanced by 16 of the 24 agents tested; others were as follows: chlortetracycline, 11; ristocetin, 8; bacitracin, 8; penicillin, 4 (see table IV).

Some explanation for the potentiating activity shown here between the compounds having plant growth activity and the antimicrobial agents might be forthcoming from reports that a number of the antibiotics influence plant growth (see review of this activity by Brian³). In addition, a number of agents having plant hormone activity have been shown to affect the rate of microbial growth and multiplication.⁴⁻⁶ It is therefore possible that a true synergistic effect is being demonstrated here.

The activity with these agents was sufficiently vivid, and many of them are of adequate low toxicity to support animal studies, which have been undertaken and are subsequently reported.

FIG. 1. Shown is the key to evaluation of synergism or antagonism on strip-gradient plate. The plate shows synergism. Bacterial growth is on the bottom.



SUMMARY

Strip-gradient plates were used to test the potentiating activity of 24 agents having plant hormone activity against *S. lutea*, *Staph. aureus*, and *Myco. tuberculosis* 607 using the antimicrobial agents penicillin, chloramphenicol, erythromycin, tetracycline, chlortetracycline, oxytetracycline, ristocetin, polymyxin B, bacitracin, isoniazid, *p*-aminosalicylic acid, pyridine-3-sulfonic acid, and streptomycin.

The agents tested as potentiators in this study proved to be the most active of any group of compounds studied to date. For example, 2,4,6-trichlorophenol, 2,4,6-trichlorophenoxyacetic acid, and α -(2,4,5-trichlorophenoxy) propionic acid showed marked synergistic activity with 11, 9, and 7 of the 13 antimicrobial agents, respectively.

ACKNOWLEDGMENTS

We are most grateful to Dr. E. E. Dunn and his colleagues at The Dow Chemical Co. for their kind cooperation in providing the phenoxy propionic acids and the phenoxy acetic acid derivatives used in this study.

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The Potentiation of Antibiotics in the Treatment of Experimental Infections

GEORGE H. SCHERR,* RITA P. NELSON, AND V. F. GERENCSEK

Department of Microbiology, The University of Illinois College of Medicine, Chicago, Ill.

In previous in vitro studies performed with strip-gradient plates,^{1,2} it was shown that a number of chemical agents having little or no therapeutic efficacy of their own could vividly enhance the antimicrobial activity of various antibiotics and other chemotherapeutic agents.

The significance of these studies was: (1) The potentiating agents (or synergists) were in general nontoxic to experimental animals. (2) Two of the four most active agents were plant hormones. (3) There was a divergency of action with the more potent synergists in that they showed activity with different antibiotics and with different organisms. Thus, α -(2,4,5-trichlorophenoxy) propionic acid (α -TPP) showed synergism with penicillin, erythromycin, bacitracin, polymyxin B, and ristocetin for *Staphylococcus aureus*, and with isoniazid and streptomycin for *Mycobacterium tuberculosis* 607.

This paper reports the successful in vivo extrapolation of these studies.

MATERIALS AND METHODS

Swiss mice, of 20 Gm. weight, which had been separated according to sex, were infected intraperitoneally with approximately 48,000 cells of a departmental culture of *Klebsiella pneumoniae*. Treatment with 10 μ g. streptomycin and/or 250 μ g. of α -TPP or 250 μ g. 2,4,6-trichlorophenol was effected by a single dose intramuscular injection with the agent(s) dissolved in 0.05 ml. of phosphate buffer.

The strain of beta-hemolytic *Streptococcus*, group A, used in this work was obtained from J. A. Hayashi of the Department of Biological Chemistry of this institution where it had been built up in pathogenicity and would kill Swiss mice.³ The organism was always harvested from frozen dead mice, which had previously been infected by the intravenous route. Such thawed mice were autopsied and fragments from the liver, spleen, or kidney, or a loopful of heart blood, were streaked on tryptose blood agar plates and incubated for 24 hours at 37 C. Beta-hemolytic colonies were picked and transferred to 5 ml. of Todd-Hewitt broth in a test tube. Such a culture, after incubation for 18 hours, could be quickly frozen and stored for use at a later date if more convenient. The Todd-Hewitt cultures were used as an inoculum in 0.1 ml. volumes for tryptose blood agar slants contained in large screw cap tubes and incubated for 18 hours at 37 C. Three ml. of sterile saline were added to each of these slants, the organisms were gently washed off the slants with a sterile loop, and pooled suspensions were centrifuged at 2000 to 2500 r.p.m. for 15 minutes. The supernatant was decanted, the cells were washed a second time, and then they were

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* Present address: Consolidated Laboratories, Inc., Chicago Heights, Ill.

TABLE I

The Potentiating Effect of α -(2,4,5-Trichlorophenoxy) Propionic Acid (α -TPP) and 2,4,6-Trichlorophenol for Streptomycin in the Treatment of Mice Infected with K. pneumoniae

Days post-infection	Number surviving mice											
	Untreated		Treated with streptomycin		Treated with streptomycin and α -TPP		Treated with streptomycin and 2,4,6-trichlorophenol		Treated with α -TPP		Treated with 2,4,6-trichlorophenol	
	M	F	M	F	M	F	M	F	M	F	M	F
1	15	15	15	15	15	15	15	15	15	15	15	15
2	15	15	15	15	15	15	15	15	15	15	15	14
3	7	6	10	8	13	13	8	13	6	6	7	6
4	5	4	9	7	12	12	8	13	4	5	4	5
5	5	3	9	7	12	11	7	13	3	5	4	5
6	5	3	9	7	12	11	7	13	3	5	4	5
7	5	3	9	7	12	11	6	13	3	5	4	4
8	4	3	9	7	12	11	5	13	3	5	4	4
9	4	3	9	7	12	11	5	13	3	5	4	4
10	4	3	9	7	12	11	5	13	3	5	4	4
11	4	3	9	7	12	10	5	13	3	5	4	3
12	4	3	9	7	12	10	5	13	3	5	4	3
13	4	3	9	7	12	10	5	13	3	5	4	3
14	4	3	9	6	12	10	5	13	3	5	4	3
15	4	3	9	6	12	10	5	13	3	5	4	3
Per cent survival at												
5 days	33.3	20	60	46	80	73	46	87	20	33.3	26	33.3
Over-all per cent survival												
at 5 days	26.7		53.3		76.7		66.7		26.7		30.0	

transferred with saline to a Kolmer centrifuge tube to which saline was added to a final volume of 10 ml. The packed cell volume was then determined so that the final suspension of organisms would result in 10 mg. of wet weight of organisms per ml. of suspension; this produced 1 mg. of wet weight per 0.1 ml. of suspension, which constituted the intravenous infecting dose for mice.

The different treatment schedules performed with penicillin were as follows: two doses of penicillin of 50 units each injected intraperitoneally; a single dose of 25 units of penicillin given by the intramuscular route; two doses of penicillin of 10 units each given by the intramuscular route. The doses of penicillin used here were deliberately chosen to be very close to the minimum necessary to elicit a small increase in survivors.

These treatment schedules were also duplicated using the same concentrations of penicillin to which had been added 1 mg. of α -TPP.

Both in the case of streptomycin and penicillin all treatment was instituted within 24 hours after infection but not earlier than four hours postinfection. If two treatment doses were administered, they were separated by a 48 hour period. Mice treated by the intramuscular route received .05 ml. of the antibiotic or the antibiotic and the synergist mixture.

Mortality curves for animals infected either with the beta-hemolytic *Streptococcus* or with the *K. pneumoniae* leveled off soon after the third day and consequently all survival data are arbitrarily here presented at the five day level.

The experiments described herein were repeated at least twice with essentially the same results as reported.

RESULTS AND DISCUSSION

Tables I and II outline the results of these experiments. It will be noted (table I) that there are differences between the sexes especially evident in the surviving mice which had been treated with the streptomycin plus the 2,4,6-trichlorophenol. It is clear that treatment solely with the α -TPP or the 2,4,6-trichlorophenol did in no way favorably alter the course of mortality of these *Klebsiella* infected mice.

There was a 33 $\frac{1}{3}$ per cent increase in survival among the male mice treated with streptomycin plus the α -TPP over those treated solely with the streptomycin; there was a 58.7 per cent increase in survival among the female mice treated with the streptomycin plus the α -TPP over those treated with streptomycin alone.

The male mice treated with the streptomycin and 2,4,6-trichlorophenol showed less survival than those treated with streptomycin alone. There is not a ready explanation for the discrepancy shown here between the male and female mice treated with

TABLE II

The Potentiating Effect of α -(2,4,5-Trichlorophenoxy) Propionic Acid (α -TPP) for Penicillin on Streptococcus Infected Mice; No. Mice Surviving Five Days after Infection/Total Infected

Sex	Untreated	Treated intraperitoneally with 2 doses of 50 I.U. penicillin each, 48 hours apart	Treated as in previous group but with 1 mg. α -TPP added
M	1/5	4/10	7/10
F	1/15	3/8	7/10
Total	2/20	7/18	14/20
Per cent surviving	10	39	70

		Treated intramuscularly with 1 dose of 25 I.U. penicillin	Treated as in previous group but with 1 mg. α -TPP added
M	3/11	2/14	5/14
F	1/16	6/15	12/15
Total	4/27	8/29	17/29
Per cent surviving	14.8	27.6	58.6

		Treated intramuscularly with 2 doses of 10 I.U. each, 48 hours apart	Treated as in previous group but with 1 mg. α -TPP added
M	3/11	5/15	6/15
F	1/16	4/15	10/15
Total	4/27	9/30	16/30
Per cent surviving	14.8	30	53.3

TABLE III

Oral Toxicity Data

Compound	100% survival	100% lethal	Animal
α -(2,4,5-Trichlorophenoxy) propionic acid	0.25 gm./Kg.	1.6 gm./Kg.	Rat
2,4,5-Trichlorophenol	1.0	2.6	Guinea pig
α -(2,4-Dichlorophenoxy) propionic acid	0.126 0.252	1.0 1.0	Rat Guinea pig
2,4-Dichlorophenol	0.3	2.0	Guinea pig
2,4,6-Trichlorophenol	2.6	>3.0	Guinea pig

streptomycin and this compound. Female mice treated with the streptomycin and the 2,4,6-trichlorophenol showed an 89 per cent increase in survival over the comparable female group treated solely with streptomycin.

It will be noted (table II) that, in general, the dosage of penicillin increased by twofold or more the per cent survivors as compared to the infected untreated animals. In every single case, the addition of 1 mg. of the potentiating agent served still further to increase significantly the percentage of survivors.

The increase in the per cent survival which can be attributed to the potentiating action of the α -TPP was in every case approximately double that found when penicillin was used alone. We have also determined from similar experiments that the potentiating agent used alone, either at this dose level of 1 mg. or at lesser or greater dose levels, had no effect in altering the course of survival of animals similarly infected.

The potentiating agent when used alone on normal uninfected mice did not appear toxic at the dose schedule utilized here. The intraperitoneal toxicity of α -TPP using a single dose has been found to be approximately 250 mg./Kg. of body weight for mice.² Oral toxicity data of the agents used here and related compounds were sent to us in correspondence by E. E. Dunn and are cited in table III because of their relevance to this study.

Since α -TPP is a plant hormone and a number of plant hormones have been shown to have an effect on the growth of microorganisms,^{4,6} it was felt necessary to determine if this potentiating agent also affected the rate of cell multiplication of the *Streptococcus*. If it were increasing the rate of multiplication, its potentiating activity might be explained by a possible increased lability to penicillin of the faster multiplying *Streptococcus*. However, direct cell counts, using a hemocytometer, on cultures grown in Todd-Hewitt broth, with and without 100 μ g./ml. of α -TPP, showed essentially identical growth curves for 51 hours of observation.

Because of the versatility of the more active synergists as already mentioned, albeit in vitro, these preliminary animal studies signify the possibility of enhancing the activity of antibiotics whose toxicity presently precludes their use at high dose levels. Such studies are now under way in our laboratory, but because of the multitude of pathogens concerned in infectious disease therapy, studies should also be undertaken by others.

SUMMARY

Experiments with mice infected with *K. pneumoniae* and treated with streptomycin indicated that the protective effect of the streptomycin, as manifest by surviving mice, could be markedly enhanced by a potentiating action of α -(2,4,5-trichlorophenoxy) propionic acid given concomitantly with the streptomycin. The potentiating agent when used alone had no protective effect on infected animals. A similar but less vivid potentiating effect was found when 2,4,6-trichlorophenol was used with the streptomycin.

Animals infected with a beta-hemolytic group A *Streptococcus* and treated with a mixture of penicillin and α -(2,4,5-trichlorophenoxy) propionic acid showed survival data that were twice as great as animals treated solely with the penicillin.

These experiments are an extension and confirmation of extensive in vitro studies with potentiators of antibiotics and suggest their possible clinical use especially in the light of their low toxicity.

ACKNOWLEDGMENT

We are most grateful to Dr. E. E. Dunn and his colleagues at The Dow Chemical Co. for their kind cooperation in providing the α -(2,4,5-trichlorophenoxy) propionic acid and 2,4,6-trichlorophenol and other helpful data and information.

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Results Obtained with Seven Different Antibiotics in Chicken Embryos Experimentally Infected with *Staphylococcus*

A. CHATTÁS, F. RAMACCIOTTI, AND R. CENTENO

*Bacteriological Laboratory of the Pediatric Department, National University of Córdoba.
Medical School, Córdoba, Argentina*

The necessity of using a more accurate method than discs to determine the antibacterial action of some antibiotics induced us to employ chicken embryos to determine their condition of development and survival, once infected with pathogenic *Staphylococcus* and later inoculated with antibiotics.

Out of 200 isolated strains in the bacteriological laboratory of the Pediatric Ward, strain number 6 was selected. This strain was isolated from a patient who had undergone an operation for severe osteomyelitis.

The strain under study was alpha-hemolytic, coagulase-positive *Staphylococcus aureus* 80/81. It showed positive gelatin liquefaction and positive mannitol fermentation. The in vitro discs method showed that this strain was sensitive to novobiocin, slightly sensitive to oleandomycin-tetracycline* and erythromycin, and resistant to penicillin, chloramphenicol, tetracycline, and chlortetracycline.

We used chicken embryos after seven days' incubation, weighing 35 Gm. and belonging to the Leghorn race, with the object of seeing their action in vivo.

TECHNIQUE

At the seventh day of incubation, we inoculated 400 *Staphylococcus* suspended in 0.2 ml. of physiological saline, via the vitelline membrane. Twenty-four hours after inoculation, they were treated with different antibiotics in concentrations of 2000 and 4000 µg. dissolved in 0.2 ml. of physiological saline. A daily check was made to observe their survival and those embryos that had died were opened and the different organs were placed in nutritive agar for recovery of the inoculated strain.

RESULTS

The results obtained with the use of the different antibiotics allowed us to verify, during this first stage of our study, that the in vivo results are different from those obtained in vitro. For better clarification, we grouped the results obtained with the seven antibiotics used, as shown in table I.

To establish the survival time of the embryos inoculated with *Staphylococcus* and not treated with antibiotics, we took 10 embryos, also with seven days' incubation, and injected them with 400 *Staphylococcus* suspended in 0.2 ml. of saline solution. Four embryos died within 24 hours and six embryos died within 48 hours.

To establish the toxicity of the antibiotics, we inoculated embryos in groups of eight for each dose of the antibiotics used in the experiment, having obtained the results outlined in table II.

* The trade name of Chas. Pfizer & Co. for oleandomycin-tetracycline is Signemycin.

TABLE I

*The Results of In Vivo Studies of Different Antibiotics on
Staphylococcus aureus Infected Chicken Embryos*

No. embryos inoculated	Dose, μ g.	Dead embryos	Alive embryos
<i>Ristocetin A and B</i>			
20	2000	2 at 48 hours 4 at 96 hours 6 at 5 days 4 at 9 days 3 at 10 days	— — — — — 1
17	4000	1 at 24 hours 4 at 96 hours 6 at 5 days 2 at 6 days 1 at 7 days 1 at 8 days 2 at 10 days	— — — — — — —
<i>Novobiocin</i>			
20	2000	2 at 24 hours 5 at 96 hours 5 at 5th day 4 at 6th day 1 at 8th day 1 at 9th day 1 at 22nd day 1 at 27th day	— — — — — — — — 1
17	4000	1 at 24 hours 6 at 96 hours 3 at 6th day 2 at 7th day 1 at 8th day 4 at 10th day	— — — — — —
<i>Oleandomycin-tetracycline</i>			
20	2000	1 at 24 hours 4 at 72 hours 7 at 96 hours 2 at 6th day 2 at 8th day 3 at 15th day	— — — — — — 1
17	4000	2 at 24 hours 5 at 96 hours 4 at 5th day 2 at 6th day 2 at 7th day 2 at 10th day	— — — — — —
<i>Oleandomycin</i>			
15	2000	4 at 48 hours 5 at 7th day 3 at 9th day 3 at 10th day	— — — —
15	4000	4 at 48 hours 8 at 7th day 2 at 9th day	— — — 1

Table I Continued on Page 328

TABLE I (Continued)

The Results of In Vivo Studies of Different Antibiotics on
Staphylococcus aureus Infected Embryos

No. embryos inoculated	Dose, μ g.	Dead embryos	Alive embryos
<i>Tetracycline</i>			
15	2000	6 at 48 hours 4 at 72 hours 3 at 7th day 2 at 9th day	— — — —
15	4000	7 at 48 hours 2 at 96 hours 4 at 5th day 2 at 7th day	— — — —
<i>Dihydrostreptomycin</i>			
15	2000	7 at 24 hours 3 at 48 hours 2 at 96 hours 2 at 5th day 1 at 7th day	— — — — —
15	4000	5 at 24 hours 7 at 96 hours 2 at 7th day 1 at 9th day	— — — —
<i>Penicillin</i>			
15	2000	6 at 24 hours 6 at 96 hours 3 at 7th day	— — —
14	4000	5 at 48 hours 4 at 96 hours 2 at 6th day 3 at 7th day	— — — —

TABLE II

Results of Toxicity Studies

Drug	No. embryos	Dose, μ g.	Average life, days
Oleandomycin-	8	2000	7
tetracycline	8	4000	5
Novobiocin	8	2000	9
	8	4000	7
Ristocetin A and B	8	2000	9
	8	4000	9
Oleandomycin	8	2000	6 to 13
	8	4000	11
Dihydrostrepto-	8	2000	13
mycin	8	4000	11
Penicillin	8	2000	12
	8	4000	10

From this investigation it is concluded that oleandomycin was the antibiotic best tolerated when used alone, that is without previous infection. It was also shown that in all the embryos infected and treated with the different antibiotics, the strain used was recoverable in the dead ones. Because of the few embryos that were finally hatched alive, it can also be concluded the most effective antibiotics were novobiocin, oleandomycin, and oleandomycin-tetracycline combination, and ristocetin A and B.

SUMMARY

A study *in vivo* was performed using 350 Leghorn chicken embryos (7 days' incubation, 35 Gm. each), inoculated with 80/81 phage type *Staph. aureus* with positive coagulase, hemolysin, gelatin liquefaction, and mannitol fermentation tests. Disc tests showed that this organism was resistant to chloramphenicol, tetracycline, and chlortetracycline; slightly sensitive to an oleandomycin-tetracycline combination and erythromycin, and sensitive to novobiocin.

Four hundred staphylococci in 0.2 ml. of saline solution were inoculated through the vitelline membrane and the embryos incubated at 37 C. The next day, 2000 or 4000 µg. of ristocetin A and B, novobiocin, an oleandomycin-tetracycline combination, oleandomycin, tetracycline, dihydrostreptomycin, or penicillin were inoculated in different groups of 15 to 20 embryos. Daily control to check survival was kept.

When the embryos died, none of the antibiotics used was able to sterilize the body tissues and staphylococci were recoverable from them.

An oleandomycin-tetracycline combination, ristocetin A and B and novobiocin were more active, comparatively. The infected embryos survived from 5 to 10 days, using these drugs. The most active was oleandomycin; embryos surviving from 5 to 15 days. With tetracycline, penicillin, and dihydrostreptomycin, the infected embryo died before the fifth day.

Twenty infected embryos were inoculated with 2000 µg. of ristocetin A and B, another 20 with 2000 µg. of an oleandomycin-tetracycline combination, and 15 with 4000 µg. of oleandomycin. In each group one embryo survived.

None of the 10 infected and nontreated embryos lived more than 48 hours.

In order to establish the tolerance to the drug we inoculated the different antibiotics (2000 or 4000 µg.) in noninfected embryos. Using oleandomycin, 4 of 16 survived. We are continuing our research using vancomycin, erythromycin, chloramphenicol, chlortetracycline, and oxytetracycline.

ACKNOWLEDGMENT

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Effect of Neomycin and Kanamycin on Bacterial Surface Sites Adsorbing Co⁶⁰ Vitamin B₁₂

ROBERT L. DAVIS, SYLVIA B. MACINTYRE, AND ALFRED H. LAWTON

Veterans Administration Center, Bay Pines, Fla.

Many microorganisms, typical of those commonly isolated from the intestinal tract, adsorb considerable quantities of certain micronutrients independent of their requirements. Earlier, we reported^{1,2} that large quantities of vitamin B₁₂ tagged with Co⁶⁰ for easy identification were readily adsorbed by many microorganisms from an environment containing this vitamin. Although organisms such as *Escherichia coli*, *Bacillus subtilis*, *Bacillus mycoides*, *Staphylococcus dobson*, among others, had considerable uptakes of Co⁶⁰ vitamin B₁₂, the lactobacilli adsorbed the greatest quantities. Both neomycin and kanamycin when administered orally are almost completely excreted in the stool in an unchanged state. Stool levels of approximately 4000 to 10,000 µg./Gm. were observed by Cohn³ after oral administrations of 1 Gm. every hour for four hours. It is of interest to determine whether these antibiotics in concentrations found in the intestinal tract during therapy exert any effect on microorganisms adsorbing vitamin B₁₂. Therefore, in vitro physical and chemical tests were performed to elucidate any mechanism whereby the bacterial disposition of vitamin B₁₂ is altered by neomycin and kanamycin. The results of such studies are described in this paper.

MATERIALS AND METHODS

Vitamin B₁₂ Co⁶⁰. The vitamin B₁₂ containing Co⁶⁰ employed in this study had a specific activity of approximately 1000 µc./mg.

The Co⁶⁰ vitamin B₁₂ employed in this study was supplied through the courtesy of Dr. Charles Rosenblum of Merck & Co., Rahway, N. J.

Cultures of *Lactobacillus leichmannii* ATCC 4797, *Lactobacillus lactis* Dorner, *E. coli* Waksman, and *E. coli* 113-3 were supplied through the courtesy of Dr. David Hendlin of Merck & Co. The concentrations of the diluted vitamin B₁₂ solutions were previously estimated by microbiological assay with *L. leichmannii* ATCC 4797 according to the method of Skeggs and co-workers.⁴

Resting Cells. The preparation of the resting cells of microorganisms investigated was described previously.⁵ The resting cells were kept at 4 to 8 C. for approximately seven days with minimum exposure to room temperature.

*Kanamycin Sulfate.** This antibiotic was prepared by dilution with distilled water or 0.85 per cent sodium chloride solution as required to yield a solution of 100 mg./ml. and served as stock solution. Further dilutions were made as required.

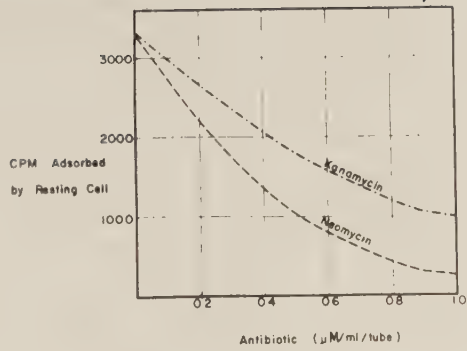
Neomycin Sulfate.† See discussion of kanamycin sulfate for preparation.

Amounts of neomycin or kanamycin as described in experimental procedures were added to resting cells standardized turbidimetrically to contain 1×10^9

* The trade name of Bristol Laboratories Inc. for kanamycin sulfate is Kantrex.

† The trade name of The Upjohn Co. for neomycin sulfate is Mycifradin sulfate.

FIG. 1. Effect of antibiotics on uptake response curve of *L. leichmannii* resting cells for Co^{60} vitamin B_{12} .



organisms in 0.85 per cent saline solution or other concentrations and salts as described in experimental detail containing 4.0 μg . vitamin B_{12} Co^{60} . The suspension was shaken thoroughly, then centrifuged. The harvested resting cells were washed with distilled water after thorough draining. The radioactivity adsorbed to the resting cells due to the vitamin B_{12} containing Co^{60} was measured with a thallium-activated sodium iodide well scintillation counter.

pH Determinations. The Beckman model G pH meter was employed for this measurement.

RESULTS

Figure 1 presents the curves derived from plotting the adsorption or uptake of a constant amount of Co^{60} vitamin B_{12} by a constant number of resting cells (1×10^9) of *L. leichmannii* against the amount ($\mu\text{M./ml.}$) of neomycin or kanamycin incorporated in the system. The curves clearly indicate that increased amounts of antibiotic result in decreased adsorption of Co^{60} vitamin B_{12} by the microorganisms. This relationship between the amount of antibiotic added and the adsorption of vitamin by the resting cells is highly reproducible and therefore can be employed to establish an assay curve for the quantitation of neomycin or any antibiotic with a similar effect.

Figure 2 shows the relationship between the adsorption of Co^{60} vitamin B_{12} by the resting cells of *L. leichmannii* with and without the incorporation of a constant amount of neomycin and the amount of Co^{60} vitamin B_{12} added. Observation of the two curves clearly indicates that neomycin will markedly reduce the adsorptive

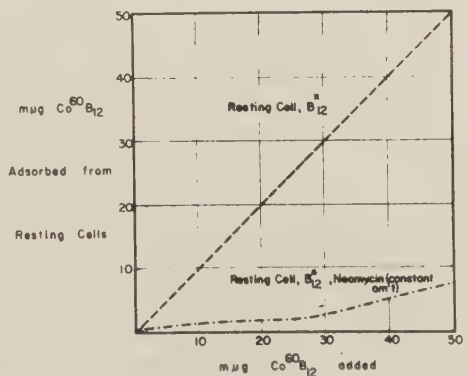


FIG. 2. Effect of increasing Co^{60} vitamin B_{12} on neomycin-resting cell system.

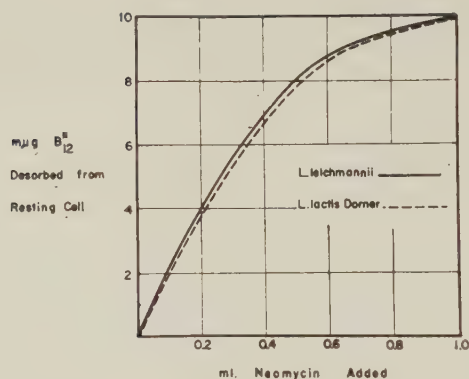


FIG. 3. Desorption of vitamin B₁₂ from resting cell with increasing amounts of neomycin.

capacity of the resting cells throughout the entire range of added radiovitamin (5 to 50 $\mu\text{g.}$). This effect is even pronounced at the 50 $\mu\text{g.}$ level when less than 20 per cent of added vitamin B₁₂ is available for adsorption by the micro-organisms. In this experiment, the resting cells of *L. leichmannii* were employed, but similar curves were obtained with *L. lactis* Dorner as well.

To determine the limiting amount of neomycin required to desorb 10 $\mu\text{g.}$ or less of Co⁶⁰ vitamin B₁₂ adsorbed to 1×10^9 resting cells, increasing amounts of neomycin were added to a constant number of resting cells previously mixed with 10 $\mu\text{g.}$ of the vitamin. The results of such a study are plotted in figure 3. The resting cells of both *L. leichmannii* and *L. lactis* Dorner were used in this experi-

TABLE I

Effect of Neomycin on Desorption and Adsorption of Large Quantities of Co⁶⁰ Vitamin B₁₂

m $\mu\text{g.}$ B ₁₂ Co ⁶⁰ added	Neomycin added	m $\mu\text{g.}$ B ₁₂ Co ⁶⁰ desorbed, resting cells (1)	m $\mu\text{g.}$ adsorbed, resting cells (2)
25	4 $\mu\text{M.}$	22.0	2
25	— (control)	<1.0	22
50	4 $\mu\text{M.}$	40.0	2
50	—	<1.0	27
75	4 $\mu\text{M.}$	55.0	
75	—	1.0	
100	4 $\mu\text{M.}$	65.0	
100	—	1.0	

Experimental Procedure

25, 50, 75, and 100 $\mu\text{g.}$ Co⁶⁰ vitamin B₁₂

Resting cells (1×10^9)

Centrifuge

Supernatant (1)

Resting cells

Resuspend in saline (0.85%)

Add 1.0 ml. neomycin (5 $\mu\text{M.}$)

Centrifuge

Supernatant (2)

Resting cells (1)

Resuspend saline

Add 25 $\mu\text{g.}$ Co⁶⁰ vitamin B₁₂

Centrifuge

Supernatant (3)

Resting cells (2)

Controls performed similarly except for neomycin

ment. The curves indicate that as little as 0.1 ml. of neomycin, equivalent to 0.1 μ M./ml. of system, can desorb or elute significant quantities of Co^{60} vitamin B_{12} from the microorganisms. At the 1.0 ml. level of neomycin, almost all of the Co^{60} vitamin B_{12} is desorbed. Both species of *Lactobacillus* containing adsorbed radio-vitamin exhibited a similar response curve to neomycin. Kanamycin exerted an effect similar to but less than that of neomycin.

Table I tabulates both the results and experimental procedure of a study on the effects of neomycin on both desorption of Co^{60} vitamin B_{12} from the resting cell and readsorption of an additional dose of Co^{60} vitamin B_{12} to such cells. In this procedure, the resting cells were treated with Co^{60} vitamin B_{12} and centrifuged. The harvested microorganisms were washed thoroughly and resuspended in 0.85 per cent sodium chloride solution. One ml. of neomycin was added to the suspension. The resting cells were again centrifuged and washed. Controls were treated similarly except for the substitution of 1 ml. saline for the neomycin. The resulting data, obtained from counting the residual Co^{60} vitamin B_{12} adsorbed on the resting cells, indicate that large quantities of this vitamin can be desorbed from the resting cells by addition of neomycin. At the 25 μ g. level of Co^{60} vitamin B_{12} previously adsorbed to the bacterial cells, approximately 90 per cent or 22 μ g. is desorbed from the cells by the incorporation of neomycin to the system. The desorption of vitamin B_{12} continues even at the 100 μ g. level, when approximately 65 per cent or 65 μ g. is detached from the microorganisms by the antibiotic. Control bacterial cells treated similarly, except for neomycin, lose little, if any, of the adsorbed Co^{60} vitamin B_{12} . When the resting cells were treated with neomycin and subsequently recentrifuged, resuspended in saline, and retreated with additional Co^{60} vitamin B_{12} , very little of the added radiovitamin was adsorbed. The control (no neomycin) adsorbed a quantity equal to the initial vitamin B_{12} adsorption. (Compare resting cell values for no. 1 and no. 2 of the control group at the 25 μ g. level of table I.)

The data indicate that the resting cells pretreated with neomycin lose their capacity to adsorb Co^{60} vitamin B_{12} . Supernatants of no. 1, 2, and 3 were likewise assayed for their Co^{60} vitamin B_{12} contents to determine both the completeness of recovery and the extent of uptake of Co^{60} vitamin B_{12} by the resting cells. The data obtained from such measurements indicate that at the 75 and 100 μ g. levels of Co^{60} vitamin B_{12} , respectively, incomplete uptake by the 1×10^9 resulting cells occurs. Therefore, additional Co^{60} vitamin B_{12} was not incorporated at these levels, and no data are presented for these respective levels in the last column of table I. Counts of resting cell and supernatant radioactivities indicated complete recovery of the added Co^{60} vitamin B_{12} .

Mutant strains of *E. coli* 113-3 and *E. coli* Waksman, which require an exogenous source of vitamin B_{12} , adsorbed Co^{60} vitamin B_{12} under experimental conditions similar to those described for the lactobacilli. However, the adsorptive capacity of the *coli* for the radiovitamin was approximately 20 per cent of that of the lactobacilli. At levels comparable to those employed for the lactobacilli, neomycin and kanamycin desorbed only about 20 per cent of the vitamin B_{12} adsorbed to the *E. coli* resting cells.

In another series of experiments, studies were performed to evaluate the effects of pH and salt concentration on the interaction of neomycin with Co^{60} vitamin B_{12}

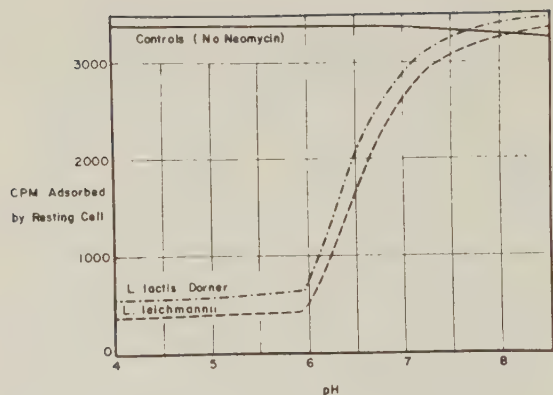


FIG. 4. Effect of pH on interaction of neomycin-vitamin B₁₂-resting cell system.

and the resting cells. Figure 4 presents graphically the Co⁶⁰ vitamin B₁₂ activity adsorbed by the resting cells of *L. leichmannii* and *L. lactis* Dorner between the pH values of 4 and 8.5 in the presence of kanamycin and neomycin respectively. It is evident from observation of the two curves that the maximum antibiotic effect occurs between pH values of 4.0 to 6.2. The antibiotics were essentially unreactive at approximately pH 7.8. At this alkaline pH, the bacterial cells adsorbed essentially equal quantities of Co⁶⁰ vitamin B₁₂ as controls receiving no antibiotic treatment. Other experiments indicated that although neomycin exerted a greater diminution of Co⁶⁰ vitamin B₁₂ adsorption than kanamycin, the curves for the two antibiotics were similar in pattern.

In view of the therapeutic concentration of approximately 4400 over 10,000 µg./Gm. stool,³ experiments were also performed to determine the extent that neomycin and kanamycin alter the stool pH. To this end, freshly collected rat stool samples were weighed and homogenized in distilled water so that each resultant aliquot contained 1 Gm. of fecal matter per 5 ml. volume. To each such aliquot, graded amounts of neomycin were added (0 to 12,000 µg.). The pH values of the neomycin-treated stool samples were obtained by measurement with a Beckman model G pH meter. The results indicate that both antibiotics lowered the pH levels approximately 0.2 to 0.3 of a pH unit, so that a fecal sample initially of pH 6.7 was lowered to pH 6.3 when an 8000 µg. of neomycin per Gm. stool level was simulated. The desorptive capacity of neomycin as well as that of kanamycin is almost at maximal capacity (fig. 4) at this pH value.

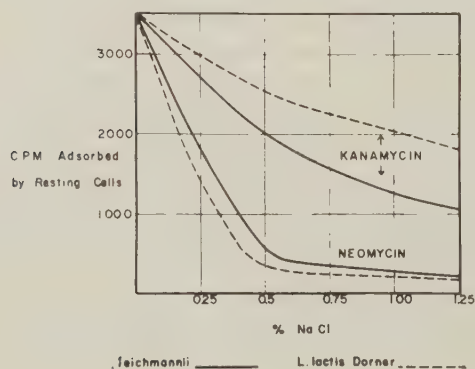


FIG. 5. Effect of salt concentration on interaction of antibiotic-vitamin B₁₂-resting cell system.

Figure 5 presents data on the salt requirements of neomycin and kanamycin for the inhibition of adsorption of Co^{60} vitamin B_{12} by the resting cells of *L. leichmannii* and *L. lactis* Dorner. The resultant curves obtained from the plot of the uptake of Co^{60} vitamin B_{12} by the resting cells expressed in counts/minute of radioactivity against the percentage of sodium chloride concentration in the presence of a constant amount of antibiotic are plotted in figure 5. The curves indicate that from approximately 0.7 to 1.25 per cent sodium chloride concentration, both antibiotics reduce significantly and almost completely the Co^{60} vitamin B_{12} uptake by the resting cells of *L. leichmannii* and *L. lactis* Dorner. When resting cells of both microorganisms were exposed to sodium chloride concentrations of 0 to 1.25 per cent and then combined with 4 μg . of Co^{60} vitamin B_{12} , no observable differences in adsorption of this vitamin were experienced throughout the entire range of salt concentration. Concentrations of sodium chloride up to 1.25 per cent, therefore, have no effect on the bacterial adsorption of Co^{60} vitamin B_{12} . The adsorptive property of the microorganisms is independent of salt and occurs equally well in distilled water. Therefore, any diminished adsorption of the Co^{60} vitamin B_{12} by the resting cells in the presence of either kanamycin or neomycin is due to the addition of antibiotics. As shown in figure 5, the data clearly demonstrate the requirement of salt for the interaction of the antibiotic, Co^{60} vitamin B_{12} , and the resting cell. To determine whether divalent cations would have similar effect on the test system, calcium chloride was employed in place of sodium chloride and investigated as described previously for sodium chloride. The results obtained indicate that calcium chloride exerted its maximal effect between levels of 0.35 (0.16 ionic strength) and 0.85 per cent, at which concentration the study was terminated. Maximal activity for sodium chloride is at approximately 0.15 ionic strength.

DISCUSSION

It has been postulated that antibiotics essentially unabsorbed and unaltered during passage through the intestinal tract act similarly to antibacterial agents *in vitro*.^{6,7} Conditions simulating the concentrations of antibiotics, pH, and ionic strength prevalent in the intestinal tract were employed in several experimental areas of this study. Since the performance of many of the *in vitro* tests of this investigation at 25 C. did not differ significantly from those performed at 37 C., the former temperature was utilized throughout.

Faloon and Fisher⁸ have reported that the normal flora can be replaced by a flora containing only lactobacilli. The lactobacilli have been demonstrated to adsorb large amounts of vitamin B_{12} . Once vitamin B_{12} is adsorbed by these microorganisms, repeated washings, dialysis, or other similar treatment does not remove appreciable amounts of this vitamin. However, when neomycin or kanamycin is added to this complex of radioactive vitamin B_{12} and resting cells under conditions outlined earlier, almost all of the Co^{60} vitamin B_{12} is desorbed or eluted rapidly by these antibiotics.

This desorptive property of neomycin and kanamycin is very much similar to that of the intrinsic factor of gastric juice, which likewise can desorb vitamin B_{12} from bacterial cells.⁹ Neomycin, however, elutes vitamin B_{12} much more rapidly from the bacterial cell than does intrinsic factor. Since neomycin and kanamycin

are administered orally in Gm. quantities for therapeutic purposes, their potential desorptive capacity is enhanced by several factors. Namely, they are neither absorbed nor materially altered by passage through intestinal tract¹⁰ and also remain in the intestinal tract until excreted. Thus large quantities of stable antibiotic are available for possible desorption of vitamin B₁₂. Dialysis experiments indicate that the vitamin B₁₂ desorbed by neomycin and kanamycin is available for absorption.

Novobiocin, which was also investigated in our laboratory, has desorptive effects similar to those of neomycin and kanamycin. There is no doubt that other antibiotics not studied would behave similarly. It well may be that other micronutrients adsorbed to certain microorganisms are desorbed by antibiotics such as neomycin and kanamycin. Further study is necessary to determine whether other systems similar to the one investigated in this report exist.

SUMMARY

1. Neomycin and kanamycin, the former to a greater extent, have the capacity to desorb large quantities of Co⁶⁰ vitamin B₁₂ previously adsorbed to the resting cells of *L. leichmannii* and *L. lactis* Dorner.

2. Both antibiotics exert their greatest influence under a slightly acidic environment and at approximately physiological salt concentrations.

3. Neomycin and kanamycin, under the experimental conditions studied, have the capacity to prevent the uptake of Co⁶⁰ vitamin B₁₂ by the resting cells or to desorb this vitamin after it has been adsorbed. Both reactions occur rapidly at 37 C. or at room temperature.

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In Vivo Study of the Combined Action of Cobalt and Various Antibiotics

PLUTARCO NARANJO AND J. CARRILLO DE MORENO

Department of Pharmacology, Central University of Ecuador, and L.I.F.E. Laboratories,
Quito, Ecuador

In the course of their studies of the mechanism of action of penicillin, Dufrenoy and Pratt¹ observed that the addition of very small amounts of cobalt to cultures of *Micrococcus pyogenes* var. *aureus* resulted in an increase in the antibacterial activity of this antibiotic. This finding has been confirmed by various other authors.²⁻⁵

In previously performed in vitro studies,⁶⁻⁸ we found, first of all, that this increase in the potency of penicillin, in regard to its action against *M. pyogenes* var. *aureus*, was much greater when penicillin-resistant strains were used than when we tested sensitive strains; a further finding was that this potentiating effect was obtained only with certain antibiotics (penicillin, dihydrostreptomycin, chloramphenicol, and oxytetracycline), whereas no change whatsoever was produced in the antibacterial effect of others (erythromycin and oleandomycin). Finally, the magnitude of the increase in potency varied from one antibiotic to another and, even for one and the same antibiotic, according to the different species and strains of bacteria.

The purpose of the present study was to establish whether cobalt produces in vivo effects similar to those observed in vitro. This study encompassed a combination of two antibiotics (tetracycline and oleandomycin*), one component of which (tetracycline) was potentiated in vitro by cobalt, whereas the other one (oleandomycin) was not, as well as a new antibiotic from Japan (colistin) that has considerable selective activity against *Escherichia coli* and other gram-negative bacteria.

MATERIALS AND METHOD

White mice, weighing 20 to 22 Gm., were used as experimental animals in this study.

The following bacteria were used: a penicillin-resistant strain of *M. pyogenes* var. *aureus*, a penicillin-resistant strain of *Streptococcus pyogenes* (hemolytic), as well as *E. coli* and *Klebsiella* sp. isolated from the urine of a patient. The bacteria were kept in solid cultures of nutrient agar (Difco), from which they were transferred to a beef broth with 1.5 per cent glucose; they were then incubated for 18 hours. The bacteria were washed subsequently by centrifugation, and, finally, we prepared a saline suspension (0.9 per cent sodium chloride) containing the desired number of bacteria per milliliter. In the computation of the number of bacteria, we used the same method as described in previous publications.⁶⁻⁸

The following antibiotics were used: potassium penicillin G, in the form of a standard solution (U.S.P. reference standard); oxytetracycline hydrochloride; a mixture of oleandomycin phosphate and tetracycline hydrochloride, in the proportion of 1:2, respectively; and colistin. All the doses of the antibiotics, except those

* The trade name of Chas. Pfizer & Co. for tetracycline-oleandomycin is Signemycin.

TABLE I

*Number of Microorganisms Required to Produce a Lethal Infection
in 50 Per Cent of the Animals (Median Lethal Dose)**

Bacteria	No. animals	LD ₅₀	Standard error
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	50	218.3×10^6	24.3×10^6
<i>Streptococcus pyogenes</i> (hemolytic)	60	258.4×10^6	31.2×10^6
<i>Escherichia coli</i>	50	4.2×10^8	0.3×10^6
<i>Klebsiella</i> sp.	50	18.5×10^6	1.3×10^6

* Bacteria were injected by the intraperitoneal route.

of colistin, were computed in terms of weights of these salts. The cobalt was used in the form of an organic compound in a colloidal solution* less toxic than cobalt chloride, but its doses were calculated as crystalline cobalt chloride.

The suspension of bacteria was introduced into the mice by intraperitoneal injection, and in the antibiotic tests, the injected amount was 0.1 ml./20 Gm. body weight for *E. coli* and *Klebsiella*, 0.4 ml./20 Gm. for *M. pyogenes*, and 0.55 ml./20 Gm. for *Str. pyogenes*. Prior to the main test of the antibiotics, preliminary tests were performed using different doses of cobalt. On the basis of results of these tests, an appropriate dose was selected for the subsequent tests, amounting to 0.5 mg./Kg. of cobalt chloride, which was kept constant in the other tests.

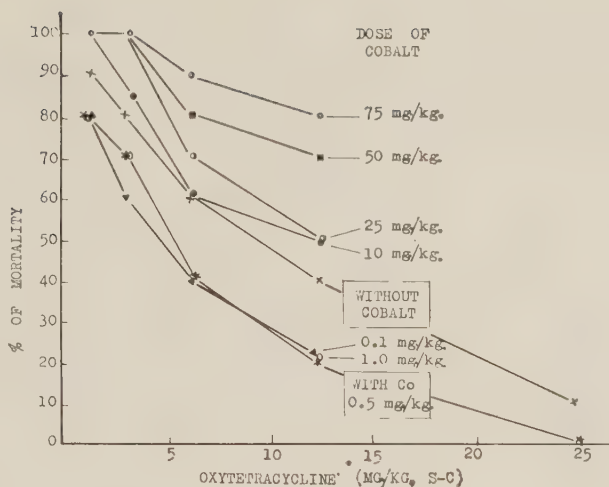
The antibiotics were injected subcutaneously, dissolved in water in the control series and in suspension with the cobalt compound in the other. The amount was constant, viz., 0.1 ml./20 Gm. body weight. Two doses were administered; the first one was given one hour after inoculation with the bacteria and the second one four hours later. The count of the surviving animals was always taken 24 hours after inoculation with the bacterial culture.

Techniques of Evaluation. We determined for each species of bacterium what quantity of the bacterial cultures had to be injected in order to produce death in 50 per cent of the mice (LD₅₀ quantity). (See table I.) For this purpose, we selected four or five appropriate "doses" of bacteria, and they were tested in groups of 10 animals. The LD₅₀ quantity and the approximate standard error were computed according to the method of Miller and Tainter.⁹

Using the same method,⁹ we determined the median effective dose (ED₅₀) of each antibiotic, which would protect 50 per cent of the mice against a dose equal to four times the LD₅₀ quantity, which exceeded the simple LD₁₀₀ quantity in all cases. Each one of the four or five doses of the antibiotic under study was tested in a group of 10 animals, with a control group for each series. One series of tests related to the antibiotic alone and another to its combination with cobalt. Using the parameters obtained, we determined the relative potency, referred in each case to the ED₅₀ quantity of the antibiotic without cobalt, and, finally, we made the respective tests of statistical significance.

* The trade name of L.I.F.E. Laboratories for cobalt organic compound is Cobaltina.

FIG. 1. The effect of doses of cobalt on mortality of white mice infected with *E. coli* and treated with oxytetracycline.



RESULTS

Effect of the Distinct Doses of Cobalt. As can be seen in figure 1, in the series of tests made with oxytetracycline, the high dose of cobalt (viz., the dose exceeding 10 mg./Kg. cobalt chloride) resulted in a diminution of the number of animals that survived the infection caused by *E. coli*, as compared with the control group which received oxytetracycline alone. On the basis of the in vitro experiments,^{7,8} according to which the high concentrations of cobalt do not increase the virulence of the microorganisms, we may assume that the lethal action of the bacteria when high doses of cobalt are given is linked with or is favored by the toxic action of the cobalt, especially since more cobalt meant a higher mortality rate as well. On the other hand, the doses below 10 mg./Kg. resulted in an increase of the survival rate—that is, in a decline of the mortality—as compared with the corresponding results obtained when the animals were treated with the antibiotic alone. This effect was no different quantitatively when we used doses between 1.0 and 0.1 mg./Kg., for which reason the dose of 0.5 mg./Kg. was selected for the subsequent experiments.

Combined Effect of Cobalt and Antibiotics. After evaluation of the activity of each antibiotic, in terms of the median effective dose that would protect the animals from death caused by the infection produced by a dose of bacteria equal to four times the LD₅₀ quantity, as shown in table II, it was found that when the animals were treated with the combination of an antibiotic and cobalt, a lesser dose of the antibiotic was required in order to obtain the same result, i.e., 50 per cent of the animals protected.

The doses of penicillin required for protection of the animals against *M. pyogenes* and *Str. pyogenes* were equal to or higher than those required for protecting them against gram-negative bacteria, owing to the fact that such bacteria belonged to strains that were resistant to penicillin.

An examination of each pair of ED₅₀ quantities, i.e., each set of such values, one of which relates to the antibiotic alone while the other relates to its combination with cobalt, indicates (table II) that the cobalt produced a certain increase in the activity of the antibiotic in all the series under study, even though the degree

TABLE II

Median Effective Dose of Some Antibiotics Protecting Against an Intraperitoneal Injection of Bacteria Equivalent to 4 LD₅₀*

Antibiotic	<i>Micrococcus pyogenes</i> var. <i>aureus</i> †		<i>Streptococcus pyogenes</i> (hemolytic)		<i>Escherichia coli</i>		<i>Klebsiella</i> sp.	
	Without cobalt	With cobalt‡	Without cobalt	With cobalt‡	Without cobalt	With cobalt‡	Without cobalt	With cobalt‡
Penicillin, u./Kg.	61.200	26.300	96.500	43.200	97.500	46.300	54.200	26.100
	± 8.300	± 3.400	± 12.300	± 5.600	± 14.400	± 4.800	± 4.700	± 3.100
Oxytetracycline, mg./Kg.	0.78	0.41	14.61	8.40	7.05	4.70	1.71	0.89
	± 0.81	± 0.36	± 1.22	± 1.43	± 1.87	± 1.4	± 0.21	± 0.07
Tetracycline-oleandomycin, mg./Kg.	1.52	0.86	10.32	6.51	17.12	11.51	3.62	2.05
	± 0.13	± 0.07	± 1.1	± 0.61	± 1.83	± 1.24	± 0.32	± 0.19
Colistin, u./Kg.	341.000	218.000	345.200	195.000	38.300	27.100	46.300	32.100
	± 28.700	± 22.400	± 36.500	± 18.900	± 3.100	± 2.400	± 5.100	± 3.600

* ED₅₀ values were calculated by using four to five groups of 10 mice each.

† Penicillin-resistant strain.

‡ Cobalt chloride, 0.5 mg./Kg.

of the increase varied according to the bacteria and the antibiotic involved. The numerical values of this increase in activity, expressed in degrees of "efficiency"¹⁰ referred to the potency of each drug, are presented in table III. Since cobalt, when applied in the dose that was used, has no antibacterial activity in and of itself, each magnitude of >1 means a superadditive effect (potentiation).

As is seen in the table, the potentiating effect was higher for penicillin, and, taking *M. pyogenes* var. *aureus* as the point of reference, it was progressively lower for oxytetracycline, colistin, and the tetracycline-oleandomycin combination.

The magnitude of potentiation for one and the same antibiotic was not very different in the four species of bacteria under study; it was, however, a little higher for the gram-positive bacteria than for the gram-negative ones, and, among the former, with the exception of colistin, it was higher for *M. pyogenes* than for *Str. pyogenes*. In the latter group it was higher for *Klebsiella* sp. with oxytetracycline and the tetracycline-oleandomycin combination, whereas it was approximately equal for both *E. coli* and *Klebsiella* in the other two antibiotics.

TABLE III

Evaluation of the Efficiency in Reference to Potency of Some Antibiotics Combined with Cobalt*

Antibiotic	<i>Micrococcus</i>		<i>Streptococcus</i>		<i>Escherichia</i>		<i>Klebsiella</i>	
	Potency	P†	Potency	P†	Potency	P†	Potency	P†
Penicillin	2.33	<0.05	2.23	<0.02	2.11	<0.02	2.07	<0.01
Oxytetracycline	1.90	<0.01	1.74	<0.05	1.50	<0.01	1.92	<0.05
Tetracycline-oleandomycin	1.80	<0.01	1.58	<0.01	1.48	<0.05	1.77	<0.01
Colistin	1.57	<0.02	1.78	<0.01	1.42	<0.01	1.44	<0.02

* Relative potency for each pair of ED₅₀ values was calculated by assigning value 1 to that of the antibiotic alone.

† Probability value of the statistical significance of the difference between the two ED₅₀ values of the antibiotic with and without cobalt.

The results obtained in the present study confirm, in general, the results that were previously obtained in vitro,^{6,8} as well as the results reported by other authors,¹⁻⁵ viz., that the combination of small amounts of cobalt with certain antibiotics increases the antibacterial activity of the latter, both in vitro and in vivo.

Certain quantitative differences are noted immediately, particularly with respect to penicillin, between the in vitro and in vivo tests. In a previous study,⁷ involving nine different strains of *M. pyogenes* var. *aureus*, potentiation of the effect of penicillin was found to vary between 3.1 and 9.3, according to the strain. In another study,⁸ dealing with various antibiotics and four species of bacteria, the potentiation of penicillin was found to be substantially higher than that of the other antibiotics; for instance, potentiation in respect to *M. pyogenes* var. *aureus* was 5.4 for penicillin, but only 1.3 and 1.5 for oxytetracycline and the tetracycline-oleandomycin combination, respectively. In the tests reported in the present paper, even though it is confirmed that the potentiation of the activity of penicillin is higher than that of the other antibiotics, the highest value obtained was 2.33, which is somewhat lower than those obtained in vitro. Likewise, the degree of potentiation of the same antibiotic in the four species of bacteria does not show in the mouse tests the same considerable difference for penicillin that was observed in vitro; it is 2.33 for *M. pyogenes* and 2.07 for *Klebsiella*, as against the 5.4 and 3.3, respectively, found in vitro.

In summary, tests in the mouse revealed a lesser potentiation of the effect of penicillin than was observed in vitro, while the potentiation was a little higher with oxytetracycline and the tetracycline-oleandomycin combination.

CONCLUSIONS

1. In tests in the mouse, small doses of cobalt produced an increase in the antibacterial activity of various antibiotics.
2. This potentiating effect of cobalt was observed in all the four antibiotics studied (penicillin, oxytetracycline, tetracycline-oleandomycin combination, and colistin) and for all the four species of bacteria used (*M. pyogenes* var. *aureus*, *Str. pyogenes*, *E. coli*, and *Klebsiella* sp.).
3. The potentiating effect was quantitatively greater for gram-positive bacteria than for gram-negative ones.
4. Among the four antibiotics, the increase in antibacterial activity was highest in penicillin (2.33 to 2.07 times) and lowest in colistin (1.78 to 1.42 times).
5. These results confirm, in general, those obtained in previous in vitro tests.

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Tyrothricin, Gramicidin, and Tyrocidine—Twenty Years Later

With Remarks on the Tissue Factors Which Affect Chemotherapy

RENÉ J. DUBOS

The Rockefeller Institute, New York, N. Y.

There are two kinds of speakers at scientific meetings—those who are eager to tell about what they have done, and those who feel the urge to express their thoughts as to what others are doing, or should be doing. My lecture today will include these two kinds of conceit, but I hope that you will see the tongue in my cheek if I appear sententious. I am old enough to know that Nature is a coquette, likely to make fools of scientists who pretend to talk in her name or to be wise about her ways. With these warnings, I feel freer to mix in this essay some recollections of the past, with speculations about the future.

Twenty years ago, I published first in the *Proceedings of the Society for Experimental Biology and Medicine*, then in the *Journal of Experimental Medicine*, a series of papers describing the separation from cultures of a spore-bearing bacillus (since identified as *Bacillus brevis*) of a soluble material active not only in vitro, but more interestingly also in vivo against a variety of bacterial species. This finding was not accidental, but rather the result of a systematic search based on a scientific philosophy inherited from Pasteur. As Pasteur repeatedly emphasized, organic matter does not accumulate in nature and microorganisms are the chief agents of its stepwise decomposition. In the light of this biological law one can assume that for any given organic substance there exists in nature some microbial agent capable of attacking it. This conviction led me in 1929 to isolate from soil new bacterial species that destroy the capsular polysaccharides of pneumococci through the agency of hydrolytic enzymes. Then in 1933 I isolated other bacteria that yielded oxidizing enzymes capable of destroying creatinine.

The findings reported in the 1939 papers, which I am reviewing today, were the results of studies aimed at finding agents capable of attacking the cell wall of staphylococci. The strain of *B. brevis* that I isolated did bring about the lysis of staphylococci, but, as it turned out, this lysis was not achieved directly. What happened was that *B. brevis* produced bactericidal substances that killed the staphylococci and thus set into activity their own autolytic enzymes. Furthermore, the active materials produced by *B. brevis* proved bactericidal not only for staphylococci, but also for many other microbial species.

I shall not recount here the chemical studies carried out in association with Rollin D. Hotchkiss which led to the demonstration that the antibacterial activity of *B. brevis* was due to two, or more, polypeptides. However, I cannot refrain from saying a few words concerning the origin of the names that we gave to these substances, especially in view of the fact that much good fun has been made of our terminology by a learned English physician.* The word tyrothricin is derived from

* It is worth noting that Dr. Vertue, who delightfully criticized our choice of the word tyrothricin, became interested in the subject after having become convinced of the merits of the substance for the treatment of diaper rash.

Tyrothrix, the genus name first given by Duclaux to the bacterial group to which *B. brevis* belongs. The name *Tyrothrix* was coined from the two Greek roots *tyro* (cheese) and *thrix* (thread), to indicate that the organism had first been isolated from cheese.* Once it was established that tyrothricin was a mixture of several active substances, we decided to call one of them gramicidin, because of its greater activity against gram-positive organisms, and the other tyrocidine to indicate the presence of free basic groups in its molecule.

Once we had in hand the purified peptides from *B. brevis*, it became possible to carry out a variety of tests to determine their biological activity both in vitro and in vivo. In the following discussion, I shall emphasize our findings with gramicidin, because this substance is the most unusual component of the tyrothricin complex, both chemically and biologically. In particular, the most extensive experience concerning the therapeutic effects against infectious processes in vivo has been obtained with gramicidin.

To speak of therapeutic effects in relation to tyrothricin and gramicidin sounds odd today, in view of the fact that there has been a tendency during recent years to regard these substances as just other examples of ordinary antiseptics—active in vitro, but ineffective in vivo. In reality, nothing could be further from the truth, and I want to spend some time on this point not merely to vindicate the good name of gramicidin and my reputation as a scientist, but also because it will serve as background for discussing some larger problems of chemotherapy. It is my intention to use what we know of the therapeutic activity of gramicidin to discuss the effect of the biochemical environment of infected tissues on the activity of other antimicrobial agents in vivo.

In experiment after experiment, under many varied conditions, it was found that a single intraperitoneal injection into mice of an incredibly small amount of tyrothricin or gramicidin (a few micrograms) was sufficient not only to prevent but even to cure a severe peritonitis caused by virulent pneumococci or by virulent group A streptococci. Needless to say, this cannot be done even with large amounts of ordinary antiseptics, such as phenolic compounds, basic dyes, or quaternary ammonium compounds. It was particularly impressive that infected animals could be saved even when tyrothricin or gramicidin were injected intraperitoneally a few hours before the expected time of death.

These findings found an immediate application in the treatment of a naturally occurring infectious process, namely, bovine mastitis caused by *Streptococcus agalactia*. In this case, control of the infection, with return to normal milk production, was achieved in a large percentage of sick animals by a few injections of tyrothricin or gramicidin directly into the infected udder through the teat canal. It might interest the reader to learn that the second herd of cows successfully treated was the one on display at the 1939–1940 Worlds Fair in New York!

While these therapeutic tests in mice and cows were going on successfully, other

* This fact is not without interest for in unpublished studies I have found that large yields of tyrothricin (or tyrothricin-like substances) can be obtained from bacterial cultures isolated from various types of cheese. In fact, I have been able to obtain the active substance itself from Oka cheese, a product of the Trappist monastery in Oka, Canada. The Trappist fathers, as well as amateurs of this most excellent North American cheese, therefore unwittingly consume fair amounts of tyrothricin when they eat the cheese.

experimental studies carried out in rabbits and dogs at the Rockefeller Institute were revealing a darker side of the picture. We soon found that tyrothricin and gramicidin lost most of their antibacterial activity when administered by the intravenous route and that, moreover, they were highly toxic under these conditions. Although the mechanisms of toxicity have never been completely unravelled, they include among others marked hemolytic effects. It is a remarkable fact that no toxic manifestation could be recognized in mice and in rabbits following repeated administration of large amounts of the substance intraperitoneally, subcutaneously, or orally; nor, for that matter, was there any evidence of therapeutic effect under these conditions. Prolonged feeding of tyrothricin to mice, guinea pigs, and chickens produced no toxic effect although it brought about a temporary change in the intestinal flora.* Clearly, tyrothricin and gramicidin are not adsorbed from these sites, or, if they are, their antibacterial and toxic activities are rapidly neutralized by tissue factors. I shall come back to these points later.

While it was obvious that tyrothricin and gramicidin could not be used for the treatment of systemic infections, the therapeutic results in mouse peritonitis and in bovine mastitis—as well as the lack of toxic effects by routes other than intravenous—left open the possibility that the substances might be useful in the treatment of certain local infections in man. In fact, this was put to the test by several groups of clinical investigators beginning in 1940. I shall mention here only three of these pioneering groups because I had personal contacts with them at that time, namely, Dr. Chester Keefer at the Boston City Hospital, Dr. Wallace Herrell, then in the Department of Medicine at the Mayo Clinic, and Dr. S. J. Crowe, then Professor of Otolaryngology at Johns Hopkins Medical School. The reports of these experienced clinical investigators, as well as of others, leave no doubt that tyrothricin and gramicidin can exert antibacterial effects, not achieved with ordinary antiseptics, in a variety of local infectious processes.

Soon, however, penicillin became widely available and for reasons that are obvious to all of us, clinical experimentation with tyrothricin and gramicidin stopped. To my knowledge, it has never been resumed on any significant scale even though it is my opinion that the chapter was closed too soon—for reasons that I shall outline later.

Before proceeding further, I shall now make a few general statements concerning antimicrobial therapy, and its fundamental limitations. Even the most enthusiastic and most naive proponents of antimicrobial therapy are now willing to admit that the prevention of, or recovery from, microbial diseases depends in final analysis upon the defense mechanisms of the body. Drugs are only adjuncts that make it possible to meet the emergency until the body takes over. At first sight, this appears like a trivial statement, which leads nowhere. In reality, however, this

* In a personal communication, Dr. Conrad Elvejehn reported to me in 1944 that animals receiving crude tyrothricin in their diet gained weight more rapidly than did the controls. He suggested at the time that this growth-promoting effect might have been due to the fact that the crude material still contained growth factors derived from the original bacterial culture. On the basis of present knowledge, it seems worth considering now that the gain in weight was similar in mechanism to that which results from feeding various antibacterial agents.

common sense view is at present the most powerful force in the experimental study of the pathogenesis of infection—witness the large research programs and the several international conferences that are being devoted to the so-called “non-specific host factors” of resistance and susceptibility to infection.

In my opinion, understanding of the role played by host factors in infection throws much light on many obscure problems of antimicrobial therapy. Antimicrobial agents, as we all know, are especially effective during the acute phases of systemic infections. In contrast, it can be said that many types of chronic infectious processes, or of localized disorders, such as tuberculous adenitis or staphylococcal abscesses, respond poorly or not at all to the drugs commonly in use. Two of the factors that may contribute to these therapeutic failures have been widely discussed, namely, the development of drug-resistant bacterial strains, and the fact that microorganisms are not susceptible to drugs when they are not in a phase of rapid multiplication, in other words when they are metabolically sluggish. I shall not discuss these points further, because they are in everybody's mind. Instead, I wish to emphasize that, in addition to these factors pertaining to the pathogen, there are other factors pertaining to the drugs themselves which in my opinion have not been sufficiently studied.

The physicochemical environment that prevails at the site of chronic abscesses or of surface infections is profoundly different from that in the circulating blood stream or other body fluids. Although no systematic study of the local microenvironment of lesions has ever been made, there are on record a large number of isolated observations that bear on the problem. Of these observations, I shall mention only a very few selected among those that deal with factors known to affect the activity of antimicrobial agents.

There is much evidence that the early phase of the inflammatory response sets in motion biochemical forces that result in marked local acidity—a state of affairs that can readily be traced to the glycolytic activity of the inflammatory cells that produce large amounts of lactic acid. Indeed, the concentration of this acid has been shown to be of the order of 0.2 per cent at certain inflammatory sites. Dye indicators agree with the results obtained by glass electrodes in showing that the acidity in these sites may reach *pH* 5.5 to 6.0. It is apparent that these conditions must limit the antibacterial activity of many drugs, in particular of those that are basic in character.

Another aspect of the local microenvironment of infectious lesions that must be considered is the presence of substances capable of interfering with antimicrobial action. It is well known that exudates are likely to contain high concentrations of albumin and low concentrations of globulin; any drug bound by albumin is therefore likely to be poorly active in exudates. The importance of this factor will be apparent to those who recall that penicillin K, so immensely active *in vitro*, proved less active than other forms of penicillin *in vivo* because of the extent to which it is bound by albumin.

In wounds as well as in repair tissues, there are exposed large amounts of acidic polysaccharides of various sorts—hyaluronic acid, chondroitin sulfuric acid, and other mucopolysaccharides; nucleic acids released by cell breakdown are also present. There is strong evidence that these large acidic molecules can bind and thus inactivate many biological agents, and antimicrobial drugs in particular. Two ex-

amples will need suffice to illustrate this point. Streptomycin forms complexes with nucleic acids and therefore loses much of its activity in situations in which these substances are released in the free state by the death of tissue cells. Sulfonamides appear inactive when tested in agar media because these drugs form complexes with the sulfuric esters of complex polysaccharides, which are the essential constituents of agar. By the same token, sulfonamides also form complexes with the polysaccharides of the ground substance, a fact that probably contributes to their ineffectiveness in wounds.

Any form of necrosis results in the local release and accumulation not only of cellular constituents released from dead tissue, but also of breakdown products resulting from autolytic changes. The early work with sulfonamides made clear that necrotic products could inhibit the antibacterial activity of these drugs, *p*-aminobenzoic acid and its derivatives being the most important inhibitor identified in this particular case. There is no doubt, furthermore, that other inhibitors exist in inflammatory sites and necrotic tissues, and thus contribute to the low activity of antimicrobial drugs in these areas.

I hardly need mention the fact that inflammatory cells, and the proper orderly sequence of tissue repair, are essential factors of recovery from infection and that any substance that interferes with these natural mechanisms of defense will prove deleterious in the long run—however effective it may be as an antimicrobial agent.* There is no doubt that the antimicrobial agents that have proved most useful in the treatment of disease are those that do not interfere either with the process of phagocytosis or with the deposition of connective repair tissue.

With these facts in mind, we can now return for a moment to the activity of gramicidin *in vivo*. I have long been puzzled by the fact that gramicidin, which is toxic and inactive as an antibacterial agent, when administered by the intravenous route, can be so immensely active against certain types of local infections. It seems to me that the facts already discussed provide some answers to the puzzle.

In unpublished experiments, we have established that gramicidin is rapidly bound by certain phospholipids. This fact certainly has unfortunate consequences because (1) blood serum contains phospholipids, which probably interfere with the antibacterial activity of gramicidin in the blood stream; (2) erythrocytes and nerve cells also contain phospholipids as an essential part of their surface structures, and it is possible therefore that the tendency of gramicidin to combine with these substances plays some part in its hemolytic activity and perhaps also in the lesions of nervous tissue that it has been reported to produce.

Granted these fundamental limitations, gramicidin possesses some unique characteristics, which differentiate it completely from ordinary antiseptics. *In vitro*, it retains all its antibacterial activity at low *pH* as well as in the presence of serum albumin and of acidic polysaccharides. It is not surprising therefore that it has proved active against certain types of surface infections, in exudates, on exposed

* In this respect, it seems to me that students of the history of chemotherapy have never given to Sir Almroth Wright the credit that he deserves. It was he who urged on Fleming the view that the tissue response was the most important factor in recovery from infection, and it was this conviction that led Fleming to use tests of toxicity for phagocytes as a guide to the selection of antibacterial agents—a practice that made him recognize promptly the potential value of penicillin.

wounds, and at inflammatory sites. Moreover, tissue culture studies have revealed that gramicidin does not interfere with the growth of animal cells, nor with their phagocytic activity. In other words, the presence of gramicidin is compatible with the operation of the normal defense mechanisms that are called into play at the site of local infections.

It seems to me that the point of view that has just been outlined might serve as a guide to reconsider some of the pessimistic philosophy concerning the treatment of local infections by antimicrobial drugs. Unfortunately, a rational and extensive discussion of this problem would demand more knowledge of the physicochemical environment prevailing at the local site of infection than is presently available. Despite the paucity of detailed information, there is enough general understanding to warrant suggesting new lines of thought and new programs of research.

As mentioned earlier, it is a fact that the antibacterial drugs in common use are most effective against the acute phases of systemic infection. In reality, this is not surprising in view of the techniques that have been used for the selection of these drugs. The universal practice is to look for substances active against pathogens at pH 7.0 to 7.4 in simple culture media free of inhibitors. These test conditions favor the discovery of agents active in the blood stream, but they almost prevent the recognition of agents capable of acting in inflammatory and necrotic areas. I wonder whether the time has not come to broaden the range of the conditions used for the screening tests both in vitro and in vivo. It might save investigators the boredom of rediscovering endlessly and uselessly substances identical or similar to those that others in the same field have discovered before them under exactly the same conditions.

I would plead also for more research on the host factors, which may play a role in inhibiting or enhancing antimicrobial therapy drugs in vivo. Here again, there is so little information available that this plea may appear but idle talk. Yet, there are several suggestive lines of approach both for experimental and for clinical work. Let me mention two examples entirely unrelated, merely as illustrations of the wide range of techniques that probably could be found to enhance the activity of different antimicrobial drugs. One of these examples is provided by the fact that the therapeutic activity of *p*-aminosalicylic acid in experimental tuberculosis can be made more evident by limiting the dietary intake of methionine. The other example concerns the enhancement of sulfonamide action against drug resistant pneumococci by simultaneous treatment of the animals with certain "chemotactically active" polysaccharide fractions.

Let me mention here that tyrothricin and gramicidin might well be used in studies focussed on the participation of host factors in the control of localized infections. While these drugs are not inhibited by the components of exudates or other tissue factors, it is also true that they do not penetrate phagocytic cells and therefore act only on bacteria that are extracellular. This situation provides a technique to investigate separately the extracellular and the intracellular aspects of local infections. Indeed, it is very likely that techniques that enhance the phagocytic activity and bactericidal power of tissue cells would be found to supplement the bacteriostatic or bactericidal action of drugs. The significance of these examples is not limited to the particular situations that I have mentioned. Stated in broader terms, the problem is to modify the host in a manner that favors or supplements drug

activity. At first sight this approach seems to be incompatible with the classical doctrine of fixity of the internal environment, but in reality—as pointed out earlier in this essay—infectious processes go on in an environment that is different from that studied by the physiologist; and there is good experimental evidence that the internal environment of infection can be modified by different techniques.

Let me acknowledge that the studies I am suggesting are difficult and expensive: Difficult because our theoretical background is so inadequate, and we know so little of the physicochemical environment in the various types of lesions and of the factors that affect the activity of natural defense mechanisms; expensive because there are many different types of lesions, each with its own microenvironment, each likely to respond differently to manipulation of host factors. Yet unless we are willing to undertake this task, it is my fear that the chemotherapy of infectious disease will come to a dead end. We can expect, of course, that some further progress will be made in the treatment of the acute phases of infection, but gilding the lilies will not long remain a rewarding occupation. The most important problems today are those concerned with the control of pathogens that do not destroy life, yet ruin it by persisting in areas of the body where known drugs are ineffective. It is obvious that gramicidin does not constitute the solution to this problem, but the fact that it retains bactericidal activity in the presence of various tissue inhibitors gives hope that other more desirable drugs will be found provided we are willing to venture outside the beaten paths of research.

Experimental and Clinical Investigations with Pyrrolidinomethyl Tetracycline

T. DIMMLING

Institute of Hygiene, University of Würzburg, Würzburg, Germany

Up to the present, the parenteral administration of antibiotics in the tetracycline series (which have proven only slightly water soluble at physiological pH values) has been impeded because of the poor tolerance of these drugs. Further, possibly because of the amphoteric nature of the substances, conversion to the salt could not produce a better tolerance for injectable preparations because these salts are only stable at extremely acid or alkaline reactions. In order to prevent side effects, if possible, intravenous forms had to be diluted and given by slow intravenous drip. For even after the intramuscular injections of small doses pain frequently occurred. Occasionally, in some cases, infiltrations and necrosis were observed. The search for drugs with greater water solubility led to the amino-methylation of tetracycline.

Pyrrolidinomethyl tetracycline* was selected as the most suitable amino-methyl compound. The main properties of the new substance proved to be the change of the iso-electric point from 4.9 (for tetracycline hydrochloride) to 7.9 (for pyrrolidinomethyl tetracycline) and remarkably improved water solubility—2500 times higher than that of tetracycline.

SPECTRUM AND INTENSITY OF EFFECTIVENESS OF PYRROLIDINOMETHYL TETRACYCLINE

A total of 227 freshly isolated strains of bacteria (*Staphylococcus aureus*, coagulase positive, α -hemolytic *Streptococcus*, β -hemolytic *Streptococcus*, *Streptococcus faecalis*, *Escherichia coli*, *Proteus vulgaris* and *Proteus mirabilis*, and *Pseudomonas aeruginosa*) were tested by the twofold dilution test, comparing pyrrolidinomethyl tetracycline with tetracycline hydrochloride. As a criterion, we selected the minimal concentration of the inhibition by reading the test tubes visually as well as by subculture on blood agar plates. Differences with regard to the effectiveness of the spectrum of both preparations were not seen in the gram-positive and gram-negative bacterias that we studied. The degree of effectiveness of pyrrolidinomethyl tetracycline was equal to that of tetracycline hydrochloride in 85 per cent of the strains tested. In 15 per cent there were small differences in sensitivity, comprising one dilution step in the series of tubes. This should be interpreted as being within the usual range of deviation of these tests. Using the turbidimetric method of semi-maximal inhibition, significant differences likewise were not seen. The effective-

* The trade name of Farbwerke Hoechst AG., Frankfurt am Main, Germany, for pyrrolidinomethyl tetracycline is Reverin.

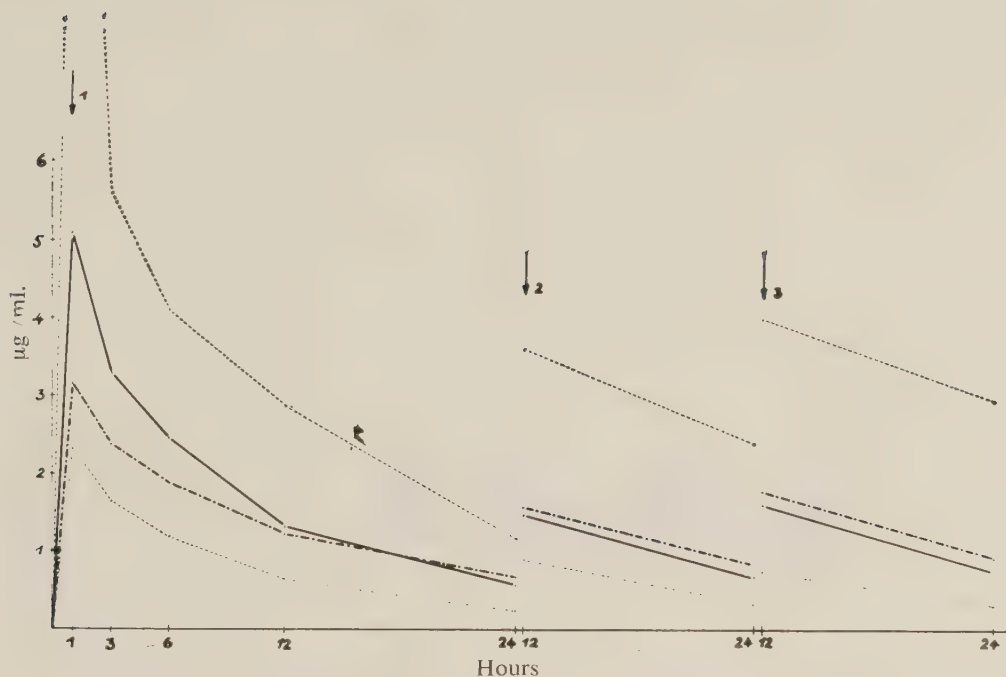


FIG. 1. Mean values of pyrrolidinomethyl tetracycline serum concentration after the first, second, and third intravenous applications in 60 patients and intramuscular application in 80 patients. Dose was 250 mg., given at intervals of 24 hours. —, intravenous; —.—, intramuscular.

ness of the spectrum and the intensity of pyrrolidinomethyl tetracycline and tetracycline hydrochloride proved to be equal.

SERUM CONCENTRATIONS AFTER INTRAVENOUS ADMINISTRATION OF PYRROLIDINOMETHYL TETRACYCLINE

Sixty surgical patients each received one intravenous injection of 250 mg. of pyrrolidinomethyl tetracycline daily over a period of several days. Determinations of the antibiotic serum levels were done at 1, 3, 6, 12, and 24 hours after the first injection and also at 12 and 24 hours after each administration on the second and third days of therapy. The cylinder test on a two layer plate with *Bacillus cereus* var. *mycoides* was used for the determination.

Figure 1 shows the mean values of all determinations. Furthermore, in order to evaluate the total range of deviation, extreme single values are given for each time a sample was taken. Also shown are the mean serum levels of 80 different patients who had received pyrrolidinomethyl tetracycline intramuscularly according to the same dosage schedule. The intravenous administration of the drug combines the advantages of high initial peak levels as well as maintaining a long-acting antibiotic concentration in the serum. This means that the intravenous administration does not have any disadvantages when compared with intramuscular, even after 12 hours. Measurable quantities of tetracycline were always found in the serum 24 hours after intravenous administration. The mean value at 24 hours with 0.56 µg./ml. is still considered to be therapeutically effective. An accumulation of the concentrations

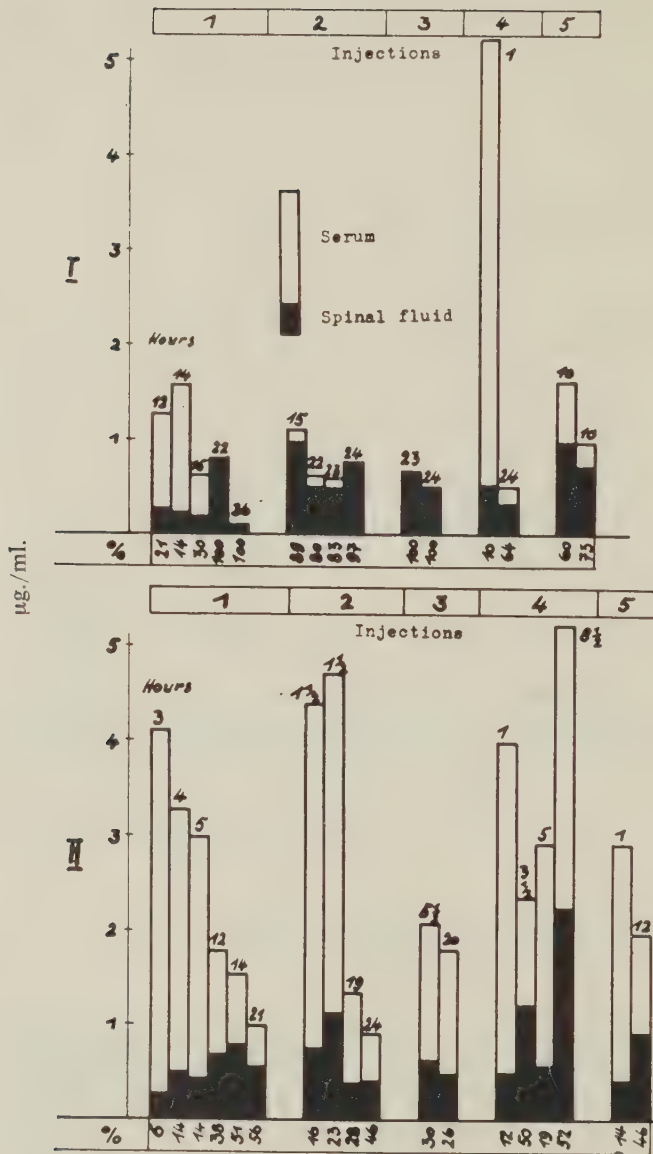


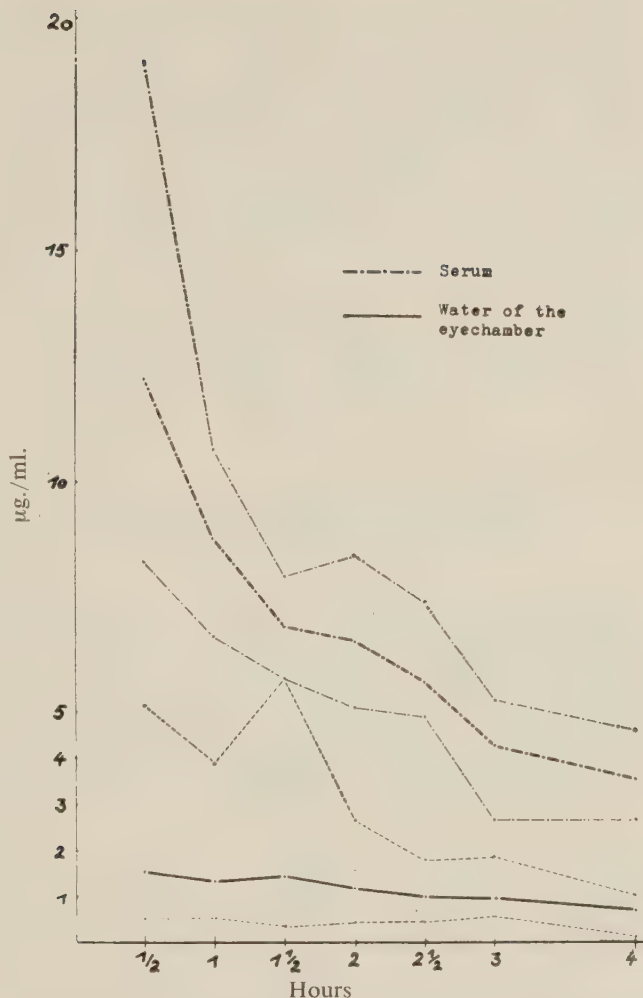
FIG. 2. Concentration of pyrrolidinomethyl tetracycline in serum and spinal fluid during the first five intravenous injections. Dose was 250 mg. Concentration in spinal fluid is expressed as per cent of the simultaneously determined serum concentration. I, patients with meningitis; II, patients without inflammatory changes.

after 12 and 24 hours could be demonstrated after the second and third intravenous administrations.

CONCENTRATIONS IN THE CEREBROSPINAL FLUID AFTER INTRAVENOUS ADMINISTRATION OF PYRROLIDINOMETHYL TETRACYCLINE

Studies were made on 45 neurosurgical patients receiving intravenous injections of 250 mg. of pyrrolidinomethyl tetracycline every 24 hours. In these very ill patients it was not possible to carry out the studies on a strict scheme. In most of the cases only one comparative determination (serum/cerebrospinal fluid) could be done at different times within the range described previously. The relation of the antibiotic concentration in the serum and the cerebrospinal fluid fluctuates, for

FIG. 3. Concentration of tetracycline in serum and aqueous humor of rabbits after intravenous administration of 10 mg./Kg. of pyrrolidinomethyl tetracycline.



increase of the concentration in the latter develops more slowly. Therefore, multiple simultaneous determinations in both media, covering long periods of time, had to be done in order to obtain sufficient information.

Our results are shown in part in figure 2. In the upper part of figure 2 are recorded determinations done on patients with meningitis. All estimations within 24 hours after intravenous administration of pyrrolidinomethyl tetracycline (also in patients without meningitis) showed measurable concentrations of tetracycline in the cerebrospinal fluid. The high serum levels after intravenous administration of the antibiotic naturally resulted in a faster increase and a higher concentration in the cerebrospinal fluid in general, when compared with intramuscular injection of equal doses.

After repeated intravenous administration of pyrrolidinomethyl tetracycline, a cumulative increase of the antibiotic in the cerebrospinal fluid is most likely. In our studies we could not find any significantly higher concentration in the cerebrospinal fluid of patients with meningitis; however, this level was greater than the simultaneously determined serum level, expressed in per cent.

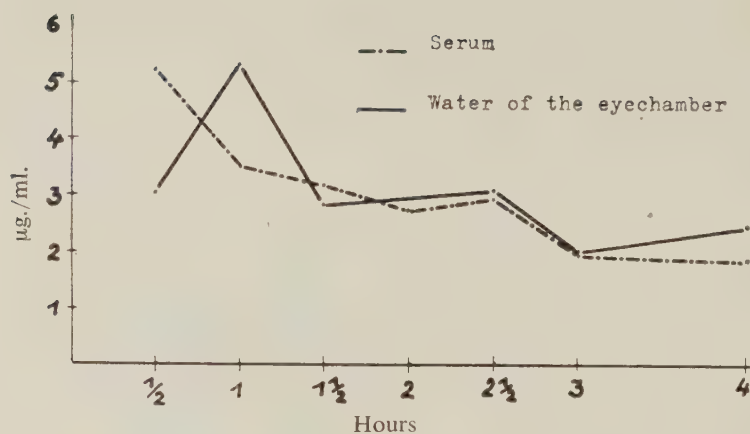


FIG. 4. Tetracycline concentration in serum and aqueous humor of rabbits after subconjunctival injection of 10 mg./Kg. of pyrrolidinomethyl tetracycline.

TETRACYCLINE CONCENTRATIONS IN THE AQUEOUS HUMOR OF THE RABBIT EYE AFTER INTRAVENOUS OR SUBCONJUNCTIVAL INJECTION OF PYRROLIDINOMETHYL TETRACYCLINE

The concentration of tetracycline in the aqueous humor of the eye after intravenous and subconjunctival injection was tested in 15 rabbits. The estimations were done in cooperation with K  hle of the ophthalmological clinic, University of Munich, who was in charge of the planning and the performance of the investigations. After preliminary experiments, the single dose given was 10 mg./Kg.

On the basis of 130 estimations and respective comparative values that have

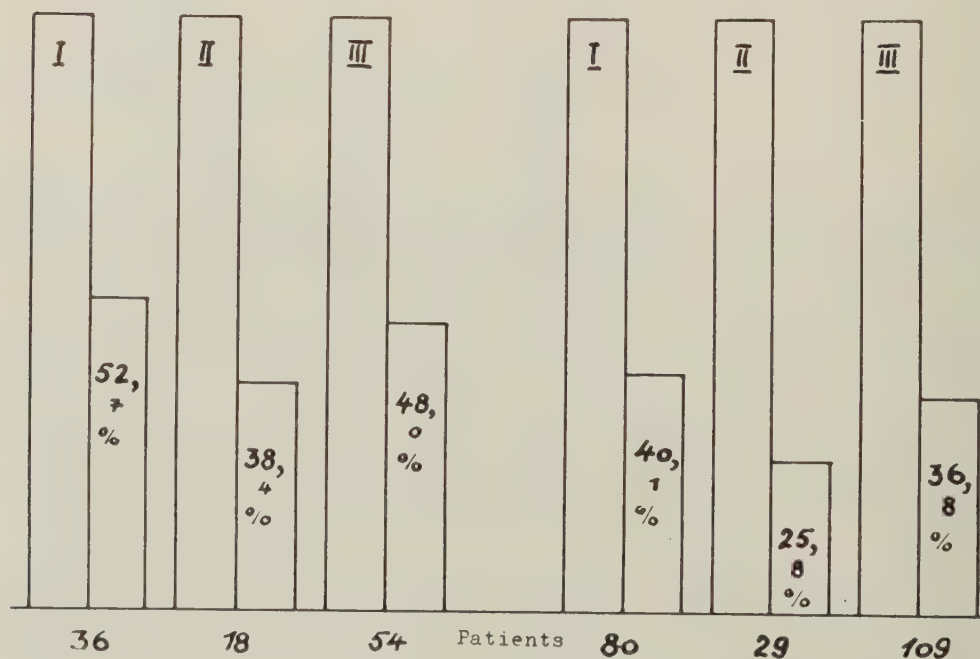


FIG. 5. Tetracycline excretion into the urine over a period of 24 hours after a single intravenous (left) and intramuscular (right) injection of 250 mg. of pyrrolidinomethyl tetracycline. I, patients without renal diseases; II, urological patients; III, all patients combined.

been done so far in the animal serum, a concentration of pyrrolidinomethyl tetracycline in the aqueous humor of the rabbit eye was observed, as is shown in figure 3. The percentage of tetracycline level in the aqueous humor of the eye is compared with the serum level. Thirty minutes after intravenous administration the serum level is 12.5 per cent and it increases to 23.1 per cent within four hours. Correspondingly higher concentrations could be obtained after a single subconjunctival injection of 10 mg. Figure 4 shows the results of 38 single determinations in the aqueous humor and a corresponding number of comparative determinations in the serum. The percentage of the mean tetracycline level in the aqueous humor compared with the mean serum level is 58.1 per cent 30 minutes after subconjunctival injection of 10 mg. of pyrrolidinomethyl tetracycline. At a later determination the tetracycline concentration in the aqueous humor is usually higher than the comparative value in the blood.

EXCRETION OF PYRROLIDINOMETHYL TETRACYCLINE INTO THE URINE AFTER INTRAVENOUS ADMINISTRATION

In 54 surgical and urological patients the excretion of pyrrolidinomethyl tetracycline into the urine after a single intravenous injection of 250 mg. was determined over a 24 hour period. In all but the urological cases, the following large tetracycline concentrations could be found in the combined samples of the collected urine during six hours: up to six hours after the injection—150 to 200 $\mu\text{g./ml.}$ on the average; between 6 and 12 hours—approximately 115 $\mu\text{g./ml.}$; up to 24 hours—more than 100 $\mu\text{g./ml.}$

The deviations of the maximal and minimal concentrations from the mean value were sometimes very distinct. This can be explained either by the remarkable variation in the urine output of the patients within the mentioned periods or by impaired renal function in the group of urological patients.

Figure 5 shows the mean values of the excreted antibiotic in per cent of the administered quantity of the antibiotic. After intravenous injection of 250 mg. of pyrrolidinomethyl tetracycline, more than half of the quantity given was excreted by patients with normal renal function. In 109 patients the total excretion rate was determined after a single intramuscular dose of 250 mg. for comparison. This excretion amounted to only 40.1 per cent of the quantity given within 24 hours.

TETRACYCLINE CONCENTRATIONS IN THE FECES OF PATIENTS AFTER INTRAVENOUS ADMINISTRATION OF PYRROLIDINOMETHYL TETRACYCLINE

In 50 surgical patients who were given intravenous injections of 250 mg. each at 24 hour intervals, the tetracycline content in the feces was determined during the first four days of therapy. In comparison with the oral medication with tetracycline hydrochloride (1 Gm./day), considerably lower concentrations of pyrrolidinomethyl tetracycline were found in the feces of these patients.

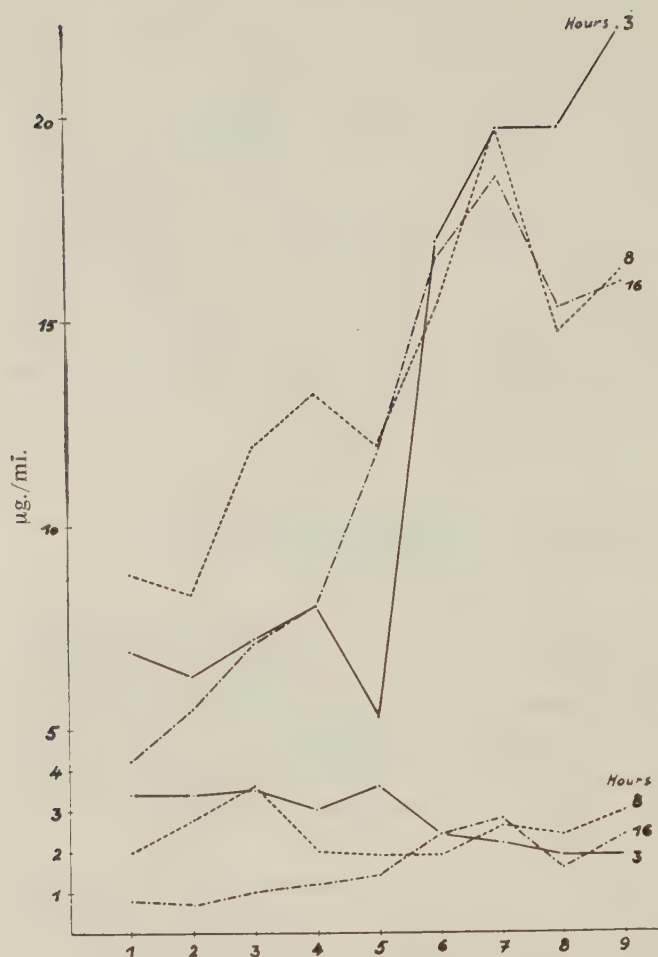


FIG. 6. Concentration of pyrrolidinomethyl tetracycline in feces of 3 rabbits after intravenous injection (bottom) or oral application (top) of 20 mg./Kg. of pyrrolidinomethyl tetracycline. Animals of each group were sacrificed 3, 8, or 16 hours after application. Numbers 1 to 9 on the abscissa correspond to the location in the intestine from which the samples were taken: 1 to 4, small bowel, each sample 30 cm. apart, starting from the pylorus; 5, zone between small bowel and beginning of appendix; 6, beginning of appendix; 7, end of appendix; 8, 30 cm. distal from appendix; 9, rectum.

TETRACYCLINE CONCENTRATIONS OF THE FECES IN VARIOUS INTESTINAL PARTS OF THE RABBIT AFTER INTRAVENOUS ADMINISTRATION OF PYRROLIDINOMETHYL TETRACYCLINE AND ORAL ADMINISTRATION OF TETRACYCLINE HYDROCHLORIDE

Three rabbits received a single intravenous dose of 20 mg. of pyrrolidinomethyl tetracycline per Kg. body weight, while 3 other animals received the same dose of tetracycline hydrochloride orally in a capsule. Three, 8, and 16 hours after the administration, 1 animal of each group was sacrificed and determinations of both tetracyclines in serum, bile, urine, and feces were done. The feces were obtained from nine different parts of the intestine, starting at the pylorus, each part 30 cm. from the next. The results are shown in figure 6.

After intravenous injection of pyrrolidinomethyl tetracycline we observed a high tetracycline concentration in serum, bile, and urine and a nearly constant level in the feces throughout the whole intestinal tract. In all 3 animals the 3 µg./ml. limit was very seldom surpassed. In the intestine of the animal sacrificed after 16 hours, the concentration of this antibiotic was only 1 µg./ml. After oral administration of tetracycline hydrochloride we found considerably higher concentrations in the feces.

Especially in the lower part of the bowel (samples 6 to 9), the concentration of the orally administered pyrrolidinomethyl tetracycline was found to be 6 to 10 times greater than after intravenous administration of the same dose of pyrrolidinomethyl tetracycline.

SUMMARY

1. Pyrrolidinomethyl tetracycline has the same spectrum as tetracycline hydrochloride.

2. Determinations of the serum concentrations in 60 patients showed that intravenous injection of 250 mg. of pyrrolidinomethyl tetracycline produced levels with a high peak. Also, 24 hours after the injection, therapeutically useful levels of the antibiotic still were present. After repeated injections of the antibiotic cumulative effects could still be observed.

3. Simultaneous determinations of intravenous pyrrolidinomethyl tetracycline in the serum and the spinal fluid of 45 patients showed that intravenous administration produces a faster increase and higher concentration of the drug than does intramuscular administration.

4. In experiments on rabbits the concentrations of pyrrolidinomethyl tetracycline in the aqueous humor and in different parts of the bowel were determined and compared with the concentrations after oral administration.

5. More than 50 per cent of the administered pyrrolidinomethyl tetracycline is excreted into the urine within 24 hours.

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Mode of Application and Therapeutic Effect of Tetracyclines in Animal Experiments

Experiences with Pyrrolidinomethyl Tetracycline

W.-H. WAGNER, W. SCHMIDT, F. BAUER, AND W. DITTMAR

Pharmaceutical Division, Farbwerke Hoechst AG., Frankfurt am Main, Germany

The method of administration of an antibiotic can influence its chemotherapeutic effect considerably. Pyrrolidinomethyl tetracycline* is a new compound of the tetracycline series that is particularly well suited for intravenous injection. Pyrrolidinomethyl tetracycline has been synthesized in the laboratories of Farbwerke Hoechst AG., Frankfurt am Main, Germany. Since 1957 it has been studied clinically, and since April, 1958, it has been widely used in Germany and many other countries after intensive investigations. This compound is readily soluble in water; its chemical properties were described by Siedel et al.²⁴ Bacteriological and pharmacological findings with pyrrolidinomethyl tetracycline were published by Dimmling et al.,⁶ Fussgänger,¹⁰ Hergott and Ther,¹⁴ Knothe,¹⁵ Marwyck,¹⁹ Rolly,²³ Strauch and Nitzschke,²⁹ Dimmling et al.,⁵ Graf and Riemann,¹² Knothe and Mahler,¹⁶ Ther et al.,³⁰ clinical results were described by Albrecht,¹ Bohn and Koch,^{2,3} Bünger et al.,⁴ Dimmling et al.,⁷ Lambrecht,¹⁷ Friederiszick and Linzenich,⁸ Fuchs and Kämmerer,⁹ Germann and Stötter,¹¹ Meyer-Rohn and Schirren,²⁰ Overkamp,²¹ Skopnik and Lückhoff,²⁶ Stenger,²⁷ Strauch and Koch,²⁸ Voiculescu et al.,³¹ Wegmann and Belser.³³

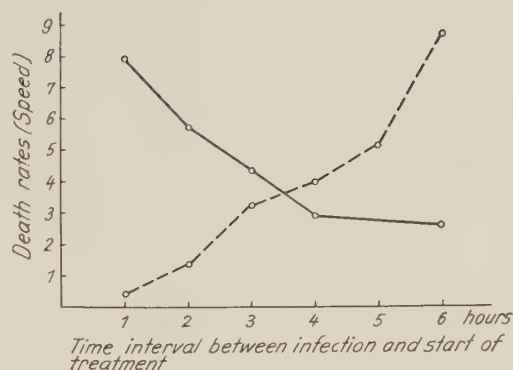
Some of these authors emphasized that the intravenous administration of this new tetracycline compound produces considerably better therapeutic results than oral administration. It is logical to look for the reason for this difference in the respective blood and tissue levels after oral or intravenous administration. To obtain a definite answer to this theory we carried out numerous experiments on mice infected with *Brucella abortus* (see also Wagner et al.³²). By this infection a high inoculation dose with a suitable strain kills the animals in a short time, whereas a low inoculation dose permits the bacteria to persist in the organism for a longer period.

EXPERIMENTAL RESULTS

In many experiments we compared oral treatment with tetracycline hydrochloride with intravenous administration of pyrrolidinomethyl tetracycline. In our first series we used a constant regimen of dosages varying the intervals between infection and treatment. One intravenous dose of pyrrolidinomethyl tetracycline was compared with four equal oral doses of tetracycline hydrochloride given every six hours, each single dose amounting to 40 mg./Kg. In all experiments we found a better effect with the latter drug given orally when treatment started immediately after or in the first hours after infection. This relation was reversed when the anti-

* The trade name of Farbwerke Hoechst AG., for pyrrolidinomethyl tetracycline is Reverin.

FIG. 1. Relation of therapeutic effect and time interval: infection—start of treatment. (Mice; *Br. abortus*; intraperitoneal infection.) o—o, tetracycline hydrochloride, 4×40 mg./Kg. orally; o—o, pyrrolidinomethyl tetracycline, 1×40 mg./Kg. intravenously.



biotics were administered three hours after infection or later. Then pyrrolidinomethyl tetracycline showed markedly better results in spite of the fact that the total dose of the orally given tetracycline hydrochloride was four times the total dose of the other drug.

In most of our experiments we used as a criterion of the therapeutic results the “speed of dying off” as determined by the formula

$$S \text{ (speed of dying off)} = \frac{1}{\text{survival time in days}} \cdot 100.^{22}$$

This value is 100 times the reciprocal value of the survival time in days. Using this formula it is possible to derive a mean value even in such cases when one animal does not die during the follow-up period (speed of dying off = 0). The more rapid the speed of dying off, the smaller is the therapeutic effect. Figure 1 shows the results of such an experiment. Each point on the curves represents the arithmetic mean of the values of S of all animals in one group of mice (the speed of dying off is inserted on the ordinate) showing its dependency from the interval between infection and start of treatment. Pyrrolidinomethyl tetracycline was given once intravenously in a dosage of 40 mg./Kg.; tetracycline hydrochloride was given orally in four doses of 40 mg./Kg. every six hours.

In further experiments we started treatment in all groups. Three hours after infection we compared the effect of a single intravenous dose of pyrrolidinomethyl tetracycline with that of one, two, three, or four equal oral doses of tetracycline hydrochloride. Based on the observed speed, we found that the single intravenous dose of the former drug was more effective than one, two, or three oral doses of the latter and showed the same effectiveness as four oral doses of the latter.

According to the rapid graphic solution of time-per cent effect curves,¹⁸ the increase of the survival time after one intravenous dose of pyrrolidinomethyl tetracycline was compared with that after one, two, three, or four oral doses of tetracycline hydrochloride (start of treatment: three hours after infection). Figure 2 shows these curves for the untreated mice and for those animals that received one dose of 40 mg./Kg. of pyrrolidinomethyl tetracycline intravenously and one or two equal doses of tetracycline hydrochloride orally. The curves for the groups treated with three or four doses of 40 mg./Kg. of the hydrochloride orally have been omitted.

The corresponding values for the ET_{50} (median effective time) are: controls:

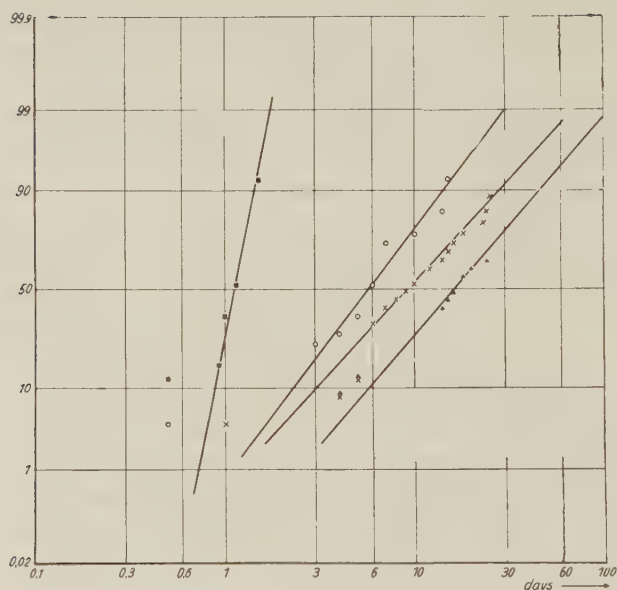


FIG. 2. Survival time of infected mice (*Br. abortus*). Start of treatment: three hours after infection. ■—■, untreated; ○—○, tetracycline hydrochloride, 1×40 mg./Kg., orally; x—x tetracycline hydrochloride, 2×40 mg./Kg., orally; ▲—▲ pyrrolidinomethyl tetracycline, 1×40 mg./Kg., intravenously.

1.1 days, pyrrolidinomethyl tetracycline; 1×40 mg./Kg. intravenously: 16.5 days, tetracycline hydrochloride; 1×40 mg./Kg. orally: 5.7 days, tetracycline hydrochloride; 2×40 mg./Kg. orally: 9.3 days, tetracycline hydrochloride; 3×40 mg./Kg. orally: 13.0 days, tetracycline hydrochloride; 4×40 mg./Kg. orally: 12.7 days. The statistical analysis of these data shows a significantly prolonged survival time of all treated groups compared with that of the controls. The ET_{50} values of pyrrolidinomethyl tetracycline and of tetracycline hydrochloride, once and twice 40 mg./Kg., differ significantly; the difference of effect between the single intravenous dose of the former and three or four oral doses of the latter could not be confirmed statistically as being significant.

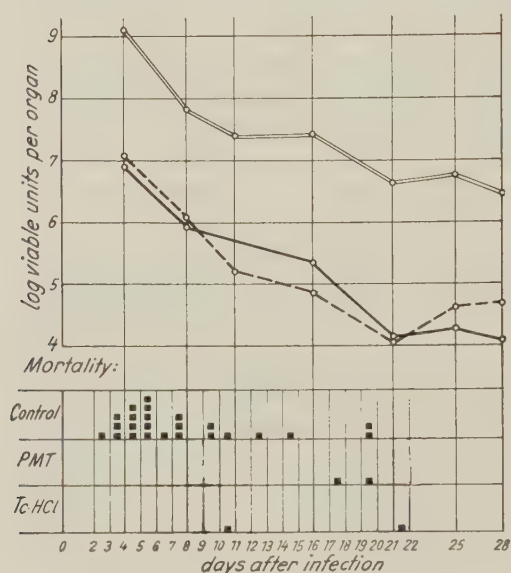
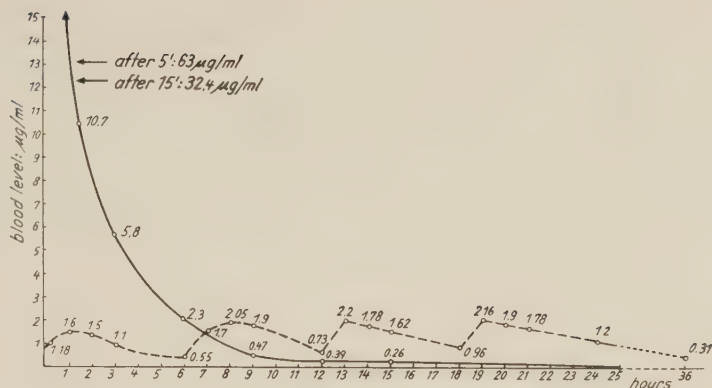


FIG. 3. Colony counts (mice; liver). Treatment: ○—○, tetracycline hydrochloride (Tc-HCl), 8×30 mg./Kg. orally at intervals of six hours; ○—○, pyrrolidinomethyl tetracycline (PMT), 2×30 mg./Kg. intravenously at intervals of 24 hours; ○—○, controls. Treatment started three hours after infection.

FIG. 4. Blood levels of tetracycline in mice. Treatment: o—o—o, tetracycline hydrochloride orally, 4×40 mg./Kg. at 0, 6, 12, and 18 hours; o—o, pyrrolidinomethyl tetracycline intravenously, 1×40 mg./Kg. at 0 hours.

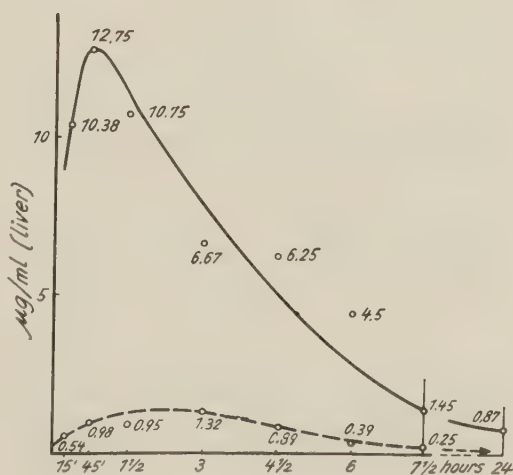


Subsequently we made colony counts from the liver and spleen of mice infected intraperitoneally with a smaller number of bacteria. In these tests we used the organs of 4 mice for each count. Here, too, we compared a single intravenous dose of pyrrolidinomethyl tetracycline with four equal oral doses of tetracycline hydrochloride. Both methods of treatment caused an obvious decrease in the number of bacteria (fig. 3). In spite of the difference of the total doses, the curves ran practically parallel. The counts of the untreated controls, too, showed a distinct decrease, but it must be considered that many of the animals had died during the first days after infection and only the resistant ones survived to the end of the experiment. The counts of bacteria in the spleen yielded corresponding results.

Summarizing our findings so far, we can state that under experimental conditions, intravenous administration of pyrrolidinomethyl tetracycline causes a therapeutic effect four times better than that of oral treatment with tetracycline hydrochloride.

To obtain an explanation for this difference we studied the blood levels of tetracycline in normal mice using 6 mice for each determination. Figure 4 shows a very marked rise of the curve after intravenous administration of pyrrolidinomethyl tetracycline. The sensitivity of our strain of *Brucella* against tetracycline was $0.26 \mu\text{g./ml.}$, and we could measure effective blood levels for 15 hours after intravenous treatment of this antibiotic. Although four oral doses of tetracycline hy-

FIG. 5. Determination of tetracycline compounds in liver tissue of mice. Treatment: o—o pyrrolidinomethyl tetracycline intravenously, 1×40 mg./Kg.; o—o—o, tetracycline hydrochloride, 1×40 mg./Kg. orally.



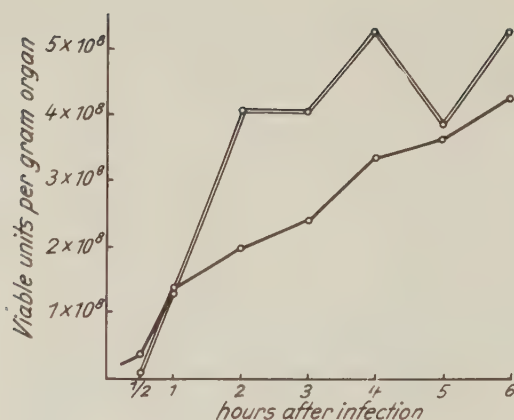


FIG. 6. Determination of viable units (*Br. abortus*) in spleen and liver of mice, after intraperitoneal infection. o==o, spleen; o—o, liver.

drochloride given every four hours maintain an effective blood level for 36 hours, the initial blood level after intravenous application exceeds by far the value measured after oral administration. Five minutes after intravenous administration of pyrrolidinomethyl tetracycline, the peak level amounts to 63 $\mu\text{g./ml.}$; for two hours the level is five times the maximum value after oral application, and after only six hours it falls below the maximum value of the oral treatment. Obviously this high initial blood level is of great importance in the therapeutic effect of intravenous pyrrolidinomethyl tetracycline treatment.

As blood levels cannot indicate sufficiently the concentration of a drug in the tissues, we continued our investigations by the determination of tissue levels. For this purpose we treated uninfected mice with 40 mg./Kg. of tetracycline hydrochloride orally or pyrrolidinomethyl tetracycline intravenously. After different intervals, 5 mice in each group were sacrificed, and the concentration of tetracycline in the organs was determined. Figure 5 is a typical example of the curves of the levels in the liver.* The particularly high concentration of pyrrolidinomethyl tetracycline after intravenous application is shown distinctly. The concentrations in the spleen showed corresponding values.

The next step was to find out why the effect of pyrrolidinomethyl tetracycline is increased when the interval between infection and treatment is extended. We studied the invasion of the organs by *Br. abortus* and found, as could be expected, that the number of bacteria in liver and spleen was growing steadily during the first hours after intraperitoneal infection (fig. 6). This finding suggests that the antibiotic is more effective when more of the bacteria have left the peritoneal cavity and invaded the organs. Under these circumstances, the high tissue level effected by intravenous administration of the drug can develop its complete effectiveness, and we think that this test method, i.e., starting treatment several hours after infection, is more comparable to clinical conditions than treatment immediately after the experimental infection would be.

SUMMARY

The results of our experiments show that the therapeutic effect of intravenous treatment with pyrrolidinomethyl tetracycline is greater than that of oral treatment

* The suspension of the organs was diluted 1:10.

with tetracycline hydrochloride proportionate to the increase in the interval between infection and treatment. When given at the height of the infection, a single intravenous dose of the former drug is more effective than one, two, or three oral doses of the latter and is equal to the effect with four oral doses of the latter. Proof of this fact was obtained by measuring the speed of dying off and by colony counts from the organs of infected mice; time-effect curves were evaluated statistically. The reasons for the considerably better results of pyrrolidinomethyl tetracycline lie in the high initial blood level and high tissue levels.

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A New Tetracycline Antibiotic for Parenteral Use

M. A. KAPLAN, H. ALBRIGHT, AND F. H. BUCKWALTER

Research Division, Bristol Laboratories Inc., Syracuse, N. Y.

Tetracycline hydrochloride and tetracycline phosphate complex* have been available for limited parenteral use in patients who are unable or unwilling to accept oral medication. These intramuscular products contain 100 or 250 mg. of tetracycline hydrochloride activity as the hydrochloride or phosphate complex,¹⁻⁴ 300 mg. of ascorbic acid, 40 mg. of procaine or lidocaine hydrochloride, and 46 mg. of anhydrous magnesium chloride, and are reconstituted with 2 to 3 ml. of water. These products have been in general use for some time and have been therapeutically effective. The tetracycline serum concentrations obtained with tetracycline hydrochloride and tetracycline phosphate complex given intramuscularly have been reported by several investigators.^{1,3,4,7-10}

A research program aimed at developing a parenteral product that would give higher serum concentrations and better efficiency of absorption and be less painful to the patient has resulted in the discovery of N-(pyrrolidinomethyl) tetracycline.^{5*} This compound is relatively less painful⁶ after injection than the parenteral forms of tetracycline hydrochloride and tetracycline phosphate complex. The tested formulation of N-(pyrrolidinomethyl) tetracycline gives serum concentrations approximately twice as high as those obtained with tetracycline hydrochloride and tetracycline phosphate complex and it is about twice as efficiently absorbed as are tetracycline hydrochloride and tetracycline phosphate complex.

The product tested by intramuscular injection, for serum concentrations, efficiency of absorption, and patient and physician acceptance, contained 350 mg. of N-(pyrrolidinomethyl) tetracycline, 40 mg. of lidocaine hydrochloride, and 300 mg. of ascorbic acid and was reconstituted with 1.7 ml. of water to give a total finished volume of 2 ml.

The product tested by intravenous administration contained 700 mg. of N-(pyrrolidinomethyl) tetracycline and 500 mg. of ascorbic acid.

N-(Pyrrolidinomethyl) tetracycline is a light yellow crystalline carboxamido condensation product of tetracycline, formaldehyde, and pyrrolidine; it melts at 160 to 165 C. The structural difference between tetracycline and N-(pyrrolidinomethyl) tetracycline is shown in figure 1.

N-(Pyrrolidinomethyl) tetracycline is stable at normal and elevated temperatures for prolonged periods of time. This product shows an average loss in potency of 15 per cent after four months at 56 C., 10 per cent after four months at 45 C., 18 per cent after 12 months at 37 C., and 7 per cent after 18 months at 25 C. The aqueous reconstituted form shows no significant loss in activity for at least one day at normal room temperature or under refrigeration.

N-(Pyrrolidinomethyl) tetracycline is extremely soluble in water, and concen-

* The trade name of Bristol Laboratories Inc. for tetracycline phosphate complex is Tetrex; for N-(pyrrolidinomethyl) tetracycline, Syntetrin.

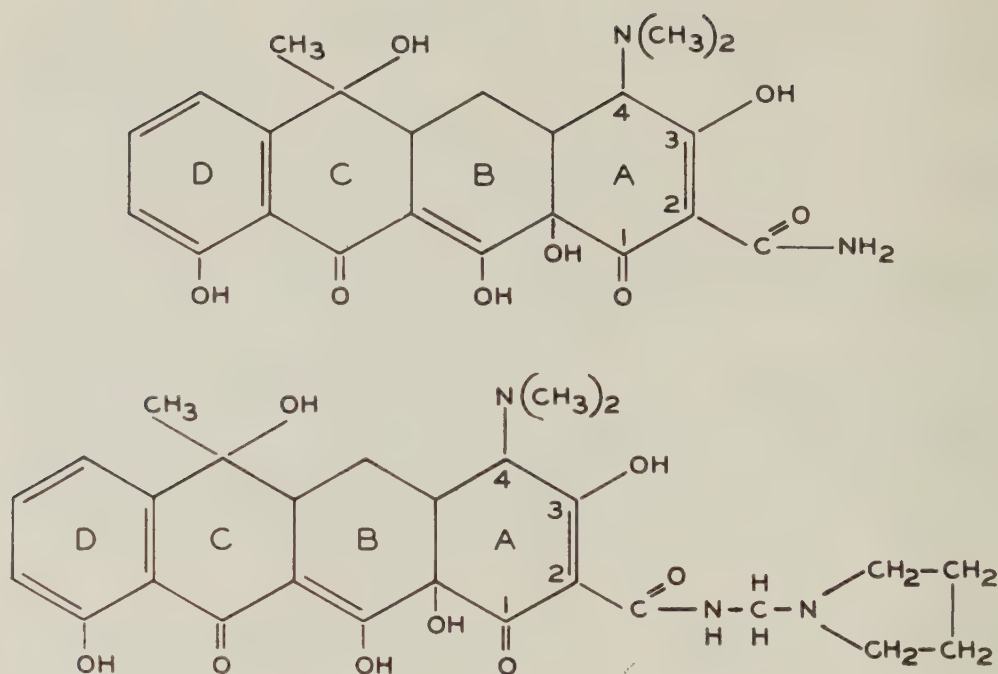


FIG. 1. The structural difference between tetracycline (top) and N-(pyrrolidinomethyl) tetracycline (bottom).

trations as high as 1 Gm. in 1 ml. of water can be readily obtained over a pH range of 1.5 to 8.5.

ABSORPTION IN HUMAN SUBJECTS

The patients were normal volunteers. Pretreatment blood specimens were taken before injection of the medication, and additional blood specimens were withdrawn at various hours (as indicated in the tables) after medication.

TABLE I

Tetracycline Serum Concentrations in Human Subjects with Intramuscular N-(Pyrrolidinomethyl) Tetracycline, Tetracycline Phosphate Complex, and Tetracycline Hydrochloride (Single Dose)

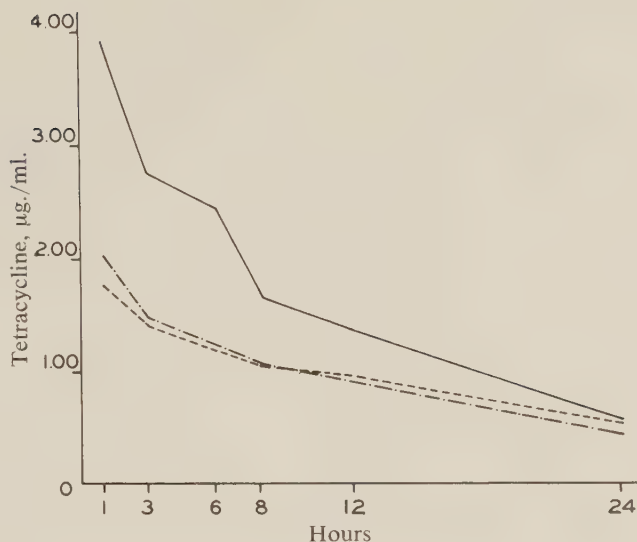
Time, hr.	N-(Pyrrolidinomethyl) tetracycline, 350 mg.*		Tetracycline phosphate complex, 330 mg.†		Tetracycline hydrochloride, 250 mg.‡	
	Number of patients	Average serum concentration, $\mu\text{g./ml.}$	Number of patients	Average serum concentration, $\mu\text{g./ml.}$	Number of patients	Average serum concentration, $\mu\text{g./ml.}$
1	114	3.94	29	1.77	5	2.04
3	40	2.76	40	1.41	5	1.48
6	10	2.45	—	—	—	—
8	45	1.66	15	1.05	5	1.06
12	44	1.37	10	0.96	—	—
24	74	0.56	46	0.54	5	0.44

* Two ml. dose equal to 250 mg. of tetracycline hydrochloride activity.

† Two ml. dose equal to 250 mg. of tetracycline hydrochloride activity.

‡ Two ml. dose equal to 250 mg. of tetracycline hydrochloride activity.

Fig. 2 Tetracycline serum concentration in human subjects. Dose equal to 2.0 ml. (250 mg.) of tetracycline hydrochloride activity. —, N-(pyrrolidinomethyl) tetracycline; — — —, tetracycline phosphate complex; — · — ·, tetracycline hydrochloride.



Tetracycline assays were performed by the cup plate method, with *Bacillus cereus* var. *mycoides* as the test organism. All sera, including the pretreatment samples, were diluted at least threefold to eliminate spurious assays. Dilution was with primary potassium phosphate buffer giving a final pH of 4.5.

Table I shows the number of patients and the average tetracycline serum concentration at each stated hour for single doses (2 ml.) of 350 mg. of N-(pyrrolidinomethyl) tetracycline, 330 mg. of tetracycline phosphate complex, and 250 mg. of tetracycline hydrochloride. All three products contained 250 mg. of tetracycline hydrochloride activity. The results are shown graphically in figure 2.

Table II shows the results of a crossover study in the same 15 patients using single doses (2 ml.) of 350 mg. of N-(pyrrolidinomethyl) tetracycline and 330 mg. of tetracycline phosphate complex. Both products contained 250 mg. of tetracycline hydrochloride activity. The results are shown graphically in figure 3.

Table III shows the number of patients and the average serum concentration of

TABLE II

Tetracycline Serum Concentrations in Human Subjects with Intramuscular N-(Pyrrolidinomethyl) Tetracycline and Tetracycline Phosphate Complex (Single Dose): Crossover Study in the Same Patients

Time, hr.	N-(Pyrrolidinomethyl) tetracycline, 350 mg.*		Tetracycline phosphate complex, 330 mg.†	
	Number of patients	Average serum concentration, µg./ml.	Number of patients	Average serum concentration, µg./ml.
0	15	0	15	0
1	15	4.9	15	1.75
3	15	2.91	15	1.29
8	15	1.95	15	0.92
24	15	0.72	15	0.62

* Two ml. dose equal to 250 mg. of tetracycline hydrochloride activity.

† Two ml. dose equal to 250 mg. of tetracycline hydrochloride activity.

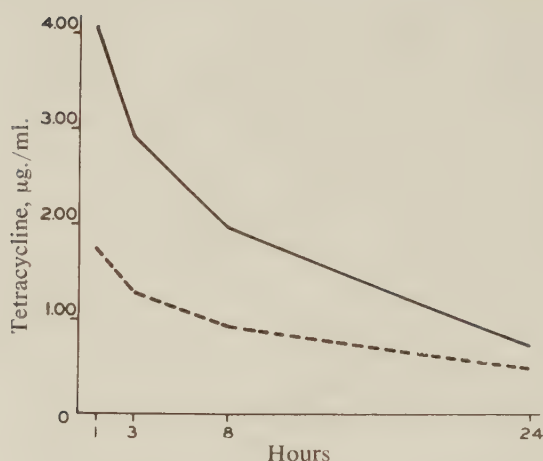


FIG. 3. Tetracycline serum concentration in human subjects after intramuscular injection of an aqueous solution. Crossover study in the same patients (15). Dose equal to 2.0 ml. (250 mg.) of tetracycline hydrochloride activity. —, N-(pyrrolidinomethyl) tetracycline; — —, tetracycline phosphate complex.

N-(pyrrolidinomethyl) tetracycline at each stated hour for single doses (2 ml.) of 350 mg. of N-(pyrrolidinomethyl) tetracycline. The results are shown graphically in figure 4.

Tables IV, V, and VI show the number of patients and the average serum concentrations of N-(pyrrolidinomethyl) tetracycline at each stated hour for single doses (2 ml.) of 350 mg. of N-(pyrrolidinomethyl) tetracycline given on multiple dose schedules of every eight hours, every 12 hours, and every 24 hours. The results are shown graphically in figures 5, 6, and 7.

Table VII shows the number of patients and the average serum concentrations of N-(pyrrolidinomethyl) tetracycline at each stated hour for 350 mg. and 700 mg. doses given intravenously using five different schedules.

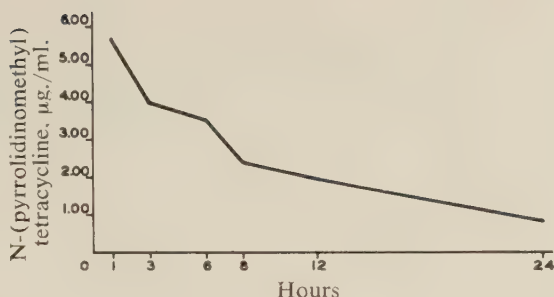
N-(Pyrrolidinomethyl) tetracycline is partially and rapidly excreted by the kidneys. Therapeutically effective urine levels are rapidly established and are well maintained after intramuscular and intravenous injection. Urine concentrations of 100 to 1000 µg. of N-(pyrrolidinomethyl) tetracycline per ml. are obtained after intramuscular injection of a 350 mg. dose. Approximately 40 to 50 per cent of the injected dose is excreted during the first 24 hours, with the remainder of the recoverable dose being excreted over the next several days.

TABLE III

*Serum Concentrations of N-(Pyrrolidinomethyl) Tetracycline in Human Subjects
(Intramuscular N-(Pyrrolidinomethyl) Tetracycline, Single Dose Schedule)
(Dose = 2 ml. = 350 mg. N-(Pyrrolidinomethyl) Tetracycline)*

Time, hr.	Number of patients	Average serum concentration, µg. of N-(Pyrrolidinomethyl) tetracycline per ml. of serum
1	114	5.63
3	40	3.94
6	10	3.50
8	45	2.37
12	44	1.96
24	74	0.79

FIG. 4. Serum concentration of N-(pyrrolidinomethyl) tetracycline in human subjects after intramuscular injection of 2.0 ml. or 350 mg. of N-(pyrrolidinomethyl) tetracycline, single dose schedule.



N-(Pyrrolidinomethyl) tetracycline is absorbed more efficiently, after intramuscular injection, than is tetracycline hydrochloride or tetracycline phosphate complex. Measurement of the area under the curves, presented in figures 2 and 3, by integration or by the use of a planimeter reveals that N-(pyrrolidinomethyl) tetracycline is more than 70 per cent better absorbed than is tetracycline hydrochloride or tetracycline phosphate complex.

TOLERANCE IN HUMAN SUBJECTS

Experiments were conducted in which each subject received simultaneous intragluteal injections of N-(pyrrolidinomethyl) tetracycline and tetracycline hydro-

TABLE IV

*Serum Concentration of N-(Pyrrolidinomethyl) Tetracycline in Human Subjects
(Intramuscular N-(Pyrrolidinomethyl) Tetracycline, Multiple Dose Schedule)
(Dose = 2 ml. = 350 mg. N-(Pyrrolidinomethyl) Tetracycline Every
8 Hours for 2 to 3 Days)*

Time, hr.	Total number of patients	Average serum concentration, µg. of N-(pyrrolidinomethyl) tetracycline per ml. of serum
1	15	4.16
8	15	1.91
9	15	6.24
16	15	3.43
17	15	7.40
24	15	4.34
25	15	9.40
32	15	5.16
33	15	9.89
40	15	5.63
41	15	11.10
48	15	5.93
49	5	10.03
56	15	5.69
57	5	8.56
64	15	5.09
65	5	10.11
72	15	5.43
80	5	3.89
88	5	2.31
96	15	1.66
120	5	0.57

TABLE V

*Serum Concentration of N-(Pyrrolidinomethyl) Tetracycline in Human Subjects
(Intramuscular N-(Pyrrolidinomethyl) Tetracycline, Multiple Dose Schedule)
(Dose = 2 ml. = 350 mg. N-(Pyrrolidinomethyl) Tetracycline Every
12 Hours for 1½ to 3 Days)*

Time, hr.	Total number of patients	Average serum concentration, μg. of N-(pyrrolidinomethyl) tetracycline per ml. of serum
1	25	4.97
3	5	3.21
8	5	2.37
12	25	2.20
13	25	7.47
24	25	2.84
25	25	7.01
36	25	3.31
37	25	9.59
48	23	3.86
49	23	9.30
60	15	3.61
61	15	8.37
72	15	3.54
96	10	0.80
120	10	0.34

chloride. Other experiments were undertaken in which a group of subjects were randomly given either medication. Subjective pain responses were recorded at the time of injection, 15 and 30 minutes, and 1, 2, 4, 8, 12, and 24 hours after injection.

Single injections of N-(pyrrolidinomethyl) tetracycline were found less painful than equivalent intramuscular doses of tetracycline hydrochloride or tetracycline phosphate complex ($p = 0.01$) when subjects received an injection of N-(pyrro-

TABLE VI

*Serum Concentration of N-(Pyrrolidinomethyl) Tetracycline in Human Subjects
(Intramuscular N-(Pyrrolidinomethyl) Tetracycline, Multiple Dose Schedule)
(Dose = 2 ml. = 350 mg. N-(Pyrrolidinomethyl) Tetracycline Every
24 Hours for 2 to 5 Days)*

Time, hr.	Total number of patients	Average serum concentration, μg. of N-(pyrrolidinomethyl) tetracycline per ml. of serum
1	39	5.99
12	9	1.50
24	39	0.60
25	39	5.36
36	8	1.43
48	38	0.93
49	38	6.07
72	30	1.19
73	30	5.67
96	30	1.19
97	15	6.41
120	15	1.23

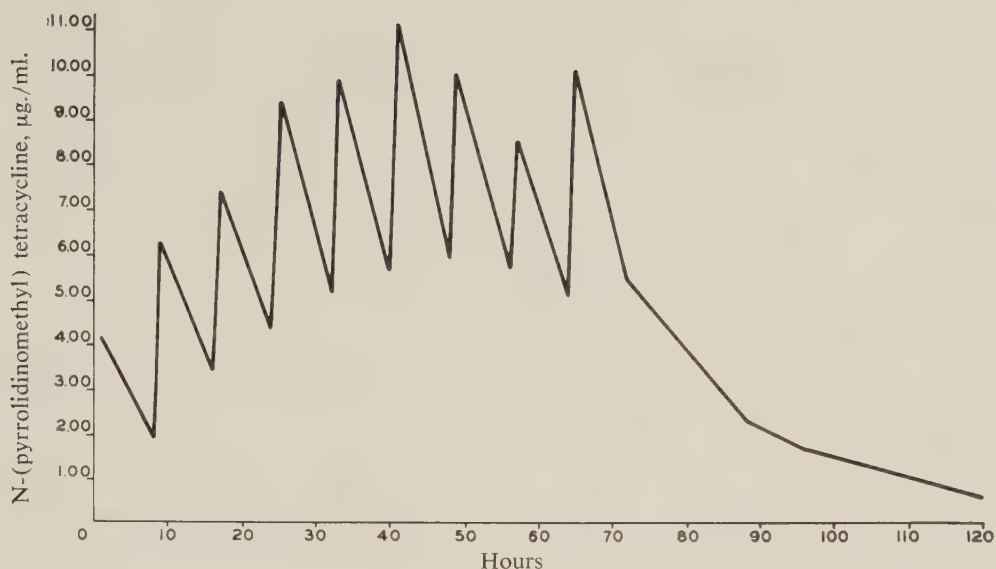


FIG. 5. Serum concentration of N-(pyrrolidinomethyl) tetracycline in human subjects after intramuscular injection of 2.0 ml. or 350 mg. of N-(pyrrolidinomethyl) tetracycline every eight hours for two to three days, multiple dose schedule.

lidinomethyl) tetracycline in one buttock and a simultaneous injection of hydrochloride or phosphate complex in the other buttock. Many subjects receiving an injection of N-(pyrrolidinomethyl) tetracycline reported discomfort, which started 10 to 15 minutes after each injection, but lasted only about 20 minutes.

A randomized group of normal subjects received either N-(pyrrolidinomethyl) tetracycline or tetracycline phosphate complex intramuscularly every 12 hours or 24 hours for five days. Those on the 24 hour schedule reported N-(pyrrolidinomethyl) tetracycline less painful than tetracycline phosphate complex ($p = 0.01$), while

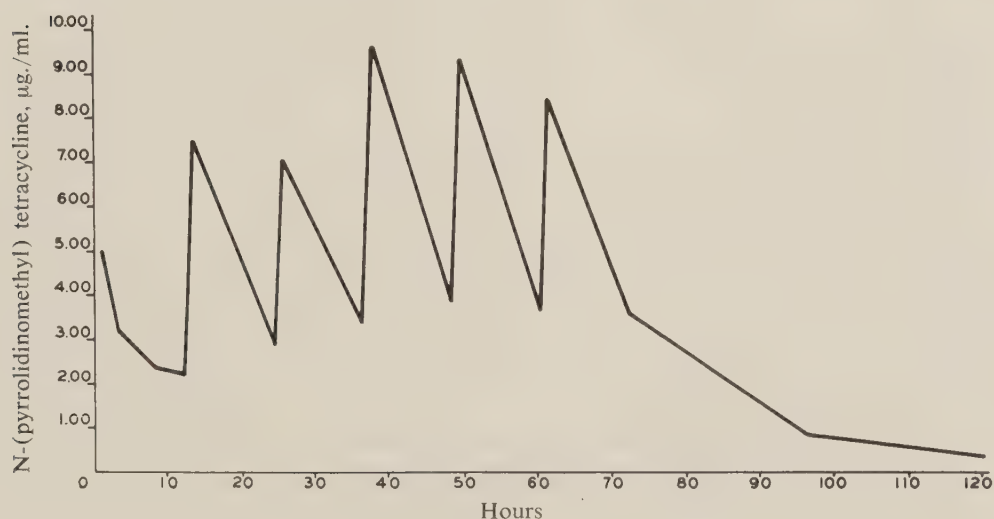


FIG. 6. Serum concentration of N-(pyrrolidinomethyl) tetracycline in human subjects after intramuscular injection of 2.0 ml. or 350 mg. of N-(pyrrolidinomethyl) tetracycline every 12 hours for one and a half to three days, multiple dose schedule.

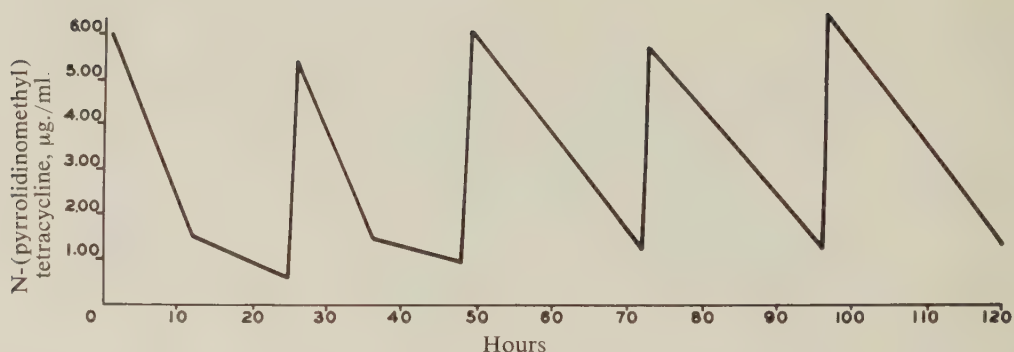


FIG. 7. Serum concentration of N-(pyrrolidinomethyl) tetracycline in human subjects after intramuscular injection of 2.0 ml. or 350 mg. of N-(pyrrolidinomethyl) tetracycline every 24 hours for two to five days, multiple dose schedule.

those on the 12 hour schedule reported no appreciable difference. Although it appeared that the pain responses were fewer with the N-(pyrrolidinomethyl) tetracycline on the 24 hour as opposed to the 12 hour schedule, these differences were not significant.

The N-(pyrrolidinomethyl) tetracycline formulation containing lidocaine hydrochloride and ascorbic acid as described herein is better tolerated than either tetracycline hydrochloride or, depending on the frequency of administration, the tetracycline phosphate complex.

TABLE VII

*Serum Concentration of N-(Pyrrolidinomethyl) Tetracycline in Human Subjects
(Intravenous N-(Pyrrolidinomethyl) Tetracycline)*

Time	Number of patients, each schedule	Average serum concentration, µg. of N-(pyrrolidinomethyl) tetracycline per ml. of serum*				
		A	B	C	D	E
5 minutes	5	21.71	—	—	—	—
10 minutes	5	—	14.43	31.14	—	—
15 minutes	5	12.89	—	—	2.94	8.17
20 minutes	5	—	—	31.00	—	—
30 minutes	5	6.97	5.86	—	4.90	9.29
40 minutes	5	—	—	13.57	—	—
1 hour	5	4.71	3.69	10.76	4.37	9.69
1 1/2 hours	5	—	—	—	4.29	11.54
2 hours	5	4.63	3.03	7.93	5.01	12.91
4 hours	5	3.69	2.86	.689	—	—
6 hours	5	—	—	—	3.44	5.60
12 hours	5	1.97	1.37	2.89	1.49	3.89
24 hours	5	0.80	0.77	1.51	0.69	1.69

* A—350 mg. of N-(pyrrolidinomethyl) tetracycline dissolved in 10 ml. of water and given intravenously in 30 to 90 seconds.

B—350 mg. of N-(pyrrolidinomethyl) tetracycline dissolved in 100 ml. of dextrose and given intravenously in 10 minutes.

C—700 mg. of N-(pyrrolidinomethyl) tetracycline dissolved in 200 ml. of dextrose and given intravenously in 20 minutes.

D—350 mg. of N-(pyrrolidinomethyl) tetracycline dissolved in 250 mg. of dextrose and given intravenously in two hours.

E—700 mg. of N-(pyrrolidinomethyl) tetracycline dissolved in 250 ml. of dextrose and given intravenously in two hours.

SUMMARY

1. A new tetracycline antibiotic, N-(pyrrolidinomethyl) tetracycline, has been described in respect to its structure, chemical and physical properties, stability, formulation, patient acceptability, and therapeutic effectiveness.

2. Serum concentrations after intramuscular injection in man indicate that this new antibiotic gives serum levels that are about twice as high as those obtained with currently available tetracycline intramuscular forms. In addition, the results indicate that N-(pyrrolidinomethyl) tetracycline is absorbed about twice as efficiently as are tetracycline hydrochloride and tetracycline phosphate complex.

3. High and adequate serum concentrations of N-(pyrrolidinomethyl) tetracycline are obtained after intravenous injection in human subjects.

4. A total of 114 volunteers received a single dose of 350 mg. (2 ml.) of N-(pyrrolidinomethyl) tetracycline by the intramuscular route. The average serum concentration of N-(pyrrolidinomethyl) tetracycline for 114 subjects at one hour was 5.63 $\mu\text{g./ml.}$; for 40 subjects at three hours, 3.94 $\mu\text{g./ml.}$; for 10 subjects at six hours, 3.50 $\mu\text{g./ml.}$; for 45 subjects at eight hours, 2.37 $\mu\text{g./ml.}$; for 44 subjects at 12 hours, 1.96 $\mu\text{g./ml.}$; and for 74 subjects at 24 hours, 0.79 $\mu\text{g./ml.}$

5. Fifteen volunteers received 350 mg. (2 ml.) of N-(pyrrolidinomethyl) tetracycline by the intramuscular route, every eight hours for two to three days. The peak serum concentrations of N-(pyrrolidinomethyl) tetracycline ranged from 4.16 $\mu\text{g./ml.}$ to 11.10 $\mu\text{g./ml.}$ and the trough serum concentrations ranged from 1.91 $\mu\text{g./ml.}$ to 5.93 $\mu\text{g./ml.}$

6. Twenty-five volunteers received 350 mg. (2 ml.) of N-(pyrrolidinomethyl) tetracycline by the intramuscular route, every 12 hours for 1½ to 3 days. The peak serum concentrations of N-(pyrrolidinomethyl) tetracycline ranged from 4.97 $\mu\text{g./ml.}$ to 9.59 $\mu\text{g./ml.}$ and the trough serum concentrations ranged from 2.20 $\mu\text{g./ml.}$ to 3.86 $\mu\text{g./ml.}$

7. Thirty-nine volunteers received 350 mg. (2 ml.) of N-(pyrrolidinomethyl) tetracycline by the intramuscular route, every 24 hours for two to five days. The peak serum concentrations of N-(pyrrolidinomethyl) tetracycline ranged from 5.36 $\mu\text{g./ml.}$ to 6.41 $\mu\text{g./ml.}$ and the trough serum concentrations ranged from 0.60 $\mu\text{g./ml.}$ to 1.23 $\mu\text{g./ml.}$

8. Effective and high urine concentrations of N-(pyrrolidinomethyl) tetracycline are achieved rapidly with doses of 350 mg. by either intramuscular or intravenous injection.

9. The 350 mg. and 700 mg. formulations of N-(pyrrolidinomethyl) tetracycline for intramuscular and intravenous use appear to be valuable additions to tetracycline therapy. Single daily intragluteal injections of 350 mg. of N-(pyrrolidinomethyl) tetracycline are better tolerated than similar injections of tetracycline hydrochloride and give serum concentrations that should be adequate for most susceptible infections.

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Observations on Demethylchlortetracycline

MAXWELL FINLAND, HANS A. HIRSCH,* AND CALVIN M. KUNIN†

*Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services,
Boston City Hospital and Department of Medicine, Harvard Medical School,
Boston, Mass.*

In August, 1957, McCormick and co-workers¹ described a new family of compounds that were closely related to the previously known tetracyclines. Two of them, namely, 6-demethyltetracycline and 7-chloro-6-demethyltetracycline, were produced by a mutant strain of *Streptomyces aureofaciens* Duggar from which chlortetracycline was originally produced. The 7-chloro-6-demethyltetracycline was subsequently given the generic name demethylchlortetracycline.‡ These two new demethylated antibiotics were found to be highly resistant to degradation by acid or alkali at relatively high temperatures and to have about the same activity as their respective methylated analogues. Preliminary studies by Sweeney and co-workers² indicated that oral administration of demethylchlortetracycline to human beings yielded more sustained levels of antibiotic in the blood than either chlortetracycline or tetracycline. In addition, demethylchlortetracycline was much more active in vitro against a test strain of *Staphylococcus aureus* than either tetracycline or oxytetracycline, although somewhat less active than chlortetracycline.

During 1957 and in the first half of 1958 there appeared a number of papers purporting to show that the absorption of tetracycline could be enhanced, i.e., that higher concentrations could be achieved and sustained in the blood by its use as a phosphate complex, and that the absorption of various tetracyclines could be enhanced by their administration together with certain additives, notably sodium hexametaphosphate, sodium metaphosphate, citric acid, and glucosamine hydrochloride. The results reported with these agents have been quite conflicting; they have been critically reviewed elsewhere^{3,4} and need not be gone into in detail here. Suffice it to say that in the course of some of these studies it was shown that many of the capsules of antibiotic that had been used in the comparisons contained dicalcium phosphate as a "filler" and this, or other calcium or magnesium salts had frequently been used by pharmacists on the assumption that they are inert. Many of the discrepancies were thus revealed as probably due to the calcium salt, which was depressing the absorption of the tetracyclines. Whatever enhancement occurred when calcium was not used was probably not sufficient to be of clinical importance, particularly in patients, because these antibiotics are given in repeated doses and in relation to food, which itself can depress absorption to a varying extent.

Aided in part by a grant (E-23) from the National Institutes of Health.

The demethylchlortetracycline used in this study was provided by the Lederle Laboratories Division, American Cyanamid Co., under the code name A-VIII.

* Present address: Munich, Germany.

† Present address: University of Virginia Medical College, Charlottesville, Va.

‡ The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin. (The trade name is Ledermycin in Canada and other countries.)

On the other hand, some of the properties of demethylchlortetracycline, as originally described, suggested that this antibiotic was worthy of exploration as a potentially superior member of the tetracycline antibiotics. Among these properties were: (1) its greater stability and resistance to degradation under conditions of elevated temperature and in both acid and alkaline media; (2) the preliminary evidence of activity comparable to the best of the earlier analogues; and (3) the early evidence that greater antibiotic activity could be attained and could be sustained longer after this agent than after tetracycline. The controversy over the blood level enhancement of tetracycline with the phosphate or by the use of adjuvants thus focused our attention on this new analogue as a possibly better and more reliable way of achieving greater antibacterial activity. Accordingly, several studies of demethylchlortetracycline and comparisons with the previous tetracyclines were undertaken and details of some of them have already been published.^{5,6}

In the first of these studies,⁵ levels of antibacterial activity in the blood following single oral doses of 500 mg. of demethylchlortetracycline hydrochloride were compared with those resulting from similar doses of chlortetracycline hydrochloride. Actually the tetracycline that was used for the comparisons contained an equal amount of citric acid, which in our own earlier studies produced levels at least as high as with any of the other preparations that had been offered.³ Two separate comparisons were made. In the first, the levels after a single oral dose, given three hours before breakfast, were compared in 8 subjects. The serums were assayed for activity against the standard assay organism, *Bacillus cereus*, both in a twofold dilution method in broth and in an agar diffusion method in which concentrations were compared with those of standard solutions of each of the antibiotics.

The results of the cruder broth dilution method showed only the much better sustained action of demethylchlortetracycline; the antibacterial activity at 24 hours was more than twice as high after this dose as after the dose of tetracycline. In the agar diffusion method when activity was based on the same standard solution of tetracycline, the average activity of serum at one hour was about the same for the two doses, but during the next 12 hours the activity resulting from demethylchlortetracycline was about two to three times that derived from tetracycline at the corresponding intervals, and at 24 hours the level was still about three times as high. Moreover, every subject still had activity in the serum 48 hours after the dose of demethylchlortetracycline, the average level at that time being greater than that found 24 hours after the doses of tetracycline. Some activity was still present in the serum of all subjects 72 hours after the doses of demethylchlortetracycline. During this study, the average half-life of demethylchlortetracycline in serum was found to be 44 per cent longer than that of tetracycline, a difference that is highly significant.

A controlled crossover study was also made of the levels of antibacterial activity reached and maintained in the serum during repeated doses of demethylchlortetracycline and tetracycline, and during normal intake of food. Each subject was given one or the other antibiotic in an initial dose of 500 mg. followed by 250 mg. every 12 hours (at 9 a.m. and 9 p.m.) for seven days, and food was taken at the usual times without restrictions. Blood specimens were obtained before and 4 and 12 hours after the morning dose on the first, second, fifth, and seventh days. After an interval of two weeks, the study was repeated, each individual receiving the alter-

nate antibiotic. All specimens from the same subject were then tested simultaneously by the broth dilution method using three standard test organisms, *Streptococcus* 98, *Staph. aureus* 209P and *B. cereus* no. 5. The results indicated that at each corresponding interval the levels of antibacterial activity in the serum against each of the three test organisms were at least twice as great at four hours and up to four or more times higher at 12 hours after the doses of demethylchlortetracycline than after the corresponding doses of tetracycline.

The serums in this study were also assayed by the agar diffusion method and concentrations compared with standard solutions of the antibiotic that was ingested. In addition, in order to obtain a more meaningful comparison of the antibiotic activities of the two antibiotics, the concentrations in the serums obtained after the doses of demethylchlortetracycline were also calculated as tetracycline activity by comparison with a standard solution of the latter. It was found that, except at four hours after the initial dose, the concentrations in terms of the antibiotic ingested were greater during the administration of demethylchlortetracycline. When all the levels after both doses were calculated as tetracycline activity, which expresses more accurately the relative activity of the two antibiotics, it is seen that at each interval, the activity derived from demethylchlortetracycline was about three to six times greater than that obtained at the corresponding interval after tetracycline. The greatest differences were observed 12 hours after the doses. Studies of renal clearances of these antibiotics carried out after the last doses showed that tetracycline was cleared by the kidney of each subject about 2.3 times as fast as demethylchlortetracycline.

In another crossover study in normal young men, the levels in serum were compared after single oral doses of 500 mg. of demethylchlortetracycline, chlortetracycline, oxytetracycline.⁵ Assays done by the broth dilution method showed that demethylchlortetracycline gave consistently higher levels of inhibitory activity in the serum than oxytetracycline against the test strains of *Staph. aureus*, hemolytic *Streptococcus* and *B. cereus*; the differences averaged 4 to 16 times as high at almost every interval. Comparisons with chlortetracycline were not done by this method because of the rapid deterioration of chlortetracycline under the condition of such assays. In the agar diffusion tests, however, when assayed as concentrations of the same antibiotic that was ingested, demethylchlortetracycline produced the highest levels, chlortetracycline the lowest, and oxytetracycline gave intermediate levels. Concentrations of the latter were similar to those of demethylchlortetracycline at one and three hours, but after that the levels of demethylchlortetracycline were much higher. However, when the activity was calculated in terms of the simultaneously run standard of any one of the four analogues, demethylchlortetracycline produced much higher levels than oxytetracycline at all intervals. At one and three hours, the activity of serum produced by demethylchlortetracycline and chlortetracycline were similar (due to the much greater activity of chlortetracycline against the test strain of *B. cereus* used), but the levels of the latter dropped rapidly after three hours while those of demethylchlortetracycline were well sustained. In comparison with the levels of activity produced by oxytetracycline, those produced by demethylchlortetracycline were several times as high at all times. Incidentally, the capsules of oxytetracycline used contained equal amounts of glucosamine hydrochloride, which is alleged to increase the levels obtainable with this antibiotic. This

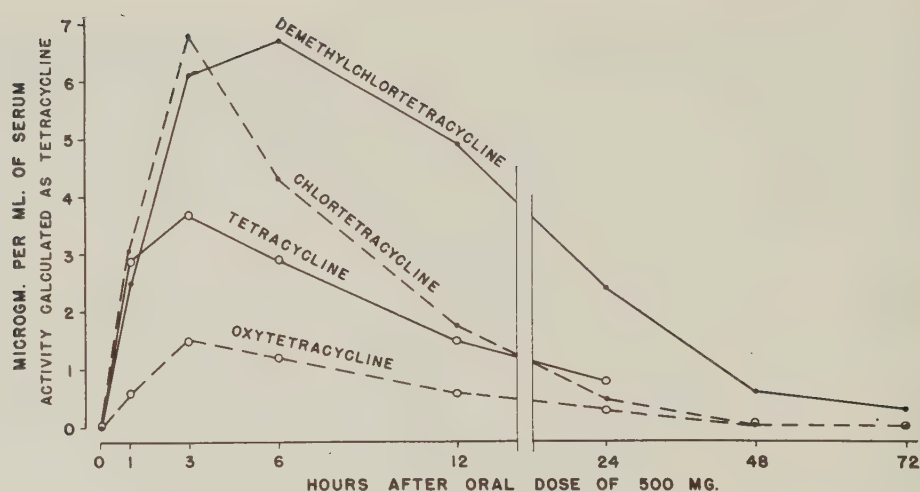


FIG. 1. Average levels of antibacterial activity, calculated as tetracycline, in blood of same subjects after single oral doses of 500 mg. of four tetracycline analogues (*Sarcina lutea*, cup-plate method).

crossover study also included a round with doses of demethylchlortetracycline given in capsules containing an equal amount of citric acid and this was shown not to influence the levels of activity produced by demethylchlortetracycline, the average levels in the same subject given this antibiotic with or without citric acid being almost identical.

A comparison of the activity of the serum after single oral doses of all four tetracyclines as determined in these studies is shown in figure 1. In this figure the levels are all expressed as equivalents of tetracycline activity. The markedly higher and more prolonged activity resulting from demethylchlortetracycline as compared with the other three analogues is clearly shown; only during the first three hours were the levels of chlortetracycline comparable.

Comparisons have also been made of the relative activity of the four tetracycline antibiotics in vitro. A total of 861 strains of 15 different genera or species of bacteria were tested in an agar plate dilution method, using appropriate nutrient media into which the antibiotics were incorporated in twofold dilutions; each strain was tested simultaneously with each of the four tetracycline analogues except 128 of the strains of *Staph. aureus* which were tested only with tetracycline and demethylchlortetracycline.

Details of all the comparisons will be presented elsewhere.⁷ However, in table I, there is shown a comparison of the activity of tetracycline and demethylchlortetracycline against all of the strains. Of interest is the difference in the relative activity of these two analogues against the sensitive strains, here defined as those in which the minimum inhibiting concentration of the more active analogue was ≤ 25 $\mu\text{g./ml.}$, and the resistant ones (those in which the minimum inhibitory concentration of the more active analogue was ≥ 50 $\mu\text{g./ml.}$). Whereas more than two thirds of the sensitive strains of *Staph. aureus* required less demethylchlortetracycline than tetracycline for complete inhibition, more than four fifths of the resistant strains of this species were susceptible to lower concentrations of tetracycline. Against nearly all other species or genera demethylchlortetracycline was more active than tetra-

cycline, although both antibiotics were generally equally active against the strains of pneumococcus, *Escherichia coli*, and the resistant strains of enterococcus. There were no other instances in which tetracycline was superior to demethylchlortetracycline. Among all the strains tested, 52 per cent were more susceptible to demethylchlortetracycline, only 18 per cent were more susceptible to tetracycline and 30 per cent were equally susceptible to both. When the strains were subdivided into the sensitive and resistant ones by the arbitrary definition given, tetracycline proved more active against nearly half of the resistant strains (one half of these strains being accounted for by the resistant staphylococci); more than one third of these resistant strains were equally susceptible to both, and only one sixth were inhibited by lower concentrations of demethylchlortetracycline. On the other hand, among the "sensitive" strains, which are probably the only ones that can be affected by therapy with these agents, demethylchlortetracycline was more active against nearly two thirds of the strains, tetracycline was more active against one in 10, and about one fourth of the strains were equally susceptible to both analogues.

TABLE I
Comparison of in Vitro Activity of Tetracycline and Demethylchlortetracycline

Organism*	No. of strains	Equal activity, %	More active	
			Tetracycline, %	Demethylchlortetra- cycline, %
<i>Staph. aureus</i> S	160	26	5	69
R	93	18	82	0
<i>D. pneumoniae</i>	25	64	12	24
<i>Str. pyogenes</i>	17	30	30	41
<i>Str. viridans</i>	20	30	30	40
Enterococcus S	33	9	6	85
R	17	65	6	29
<i>N. gonorrhoeae</i>	132	25	3	72
<i>N. meningitidis</i>	15	33	27	40
<i>H. influenzae</i>	50	28	10	62
<i>E. coli</i> S	67	51	24	25
R	13	85	15	0
<i>Klebsiella</i>	74	16	10	74
<i>Aerobacter</i> S	37	22	8	70
R	3	1†	1	1
<i>Shigella</i> S	28	32	7	61
R	2	—	1	1
<i>Salmonella</i> S	18	28	0	72
R	1	1	—	—
<i>Proteus</i> S	4	1	—	3
R	28	36	11	54
<i>Pseudomonas</i> R	24	58	13	29
Total "sensitive"	680	28	10	62
Total "resistant"	181	36	48	16
Total	861	30	18	52

* S = sensitive (minimum inhibitory concentration = ≤25); R = resistant (minimum inhibitory concentration = ≥50) for the more active analogue.
 † Italics indicate number of strains when total is <10.

To simplify the comparisons among all four antibiotics, comparative ratings were given to the activity of each analogue in comparison with the most active one, which was given a rating of 1; an additional unit was added for each twofold concentration of the other antibiotic required to produce complete inhibition. The average ratings of the four analogues for the 733 strains that were tested with all four analogues are shown in table II. This also shows the percentage of strains against which each antibiotic was most active; when two or more analogues were equally active, but more active than the others, they are included in the percentage of the most active agent. The superiority of demethylchlortetracycline is shown against about two thirds of the "sensitive" strains, whereas tetracycline appears superior to the other analogues against about one half of the strains. The differences in the average ratings also reflect the same order of activity, the lowest ratings representing the most active analogue. The small differences in these ratings, however, indicate that, in general, the activity of all of the analogues is appreciably different, but quantitatively the differences, on the average, are not very striking.

Concurrent comparisons of the clinical effectiveness of demethylchlortetracycline and tetracycline and of the untoward effects resulting from their use have been carried out during the past few months at the Boston City Hospital on the adult medical wards of the Harvard Medical Services. This comparison was conducted in a manner identical to that used in a previous comparison of chlortetracycline and oxytetracycline,⁸ except as to the dosages employed. Some wards were provided with capsules each of which contained 250 mg. of tetracycline hydrochloride and alternate wards were given capsules containing 125 mg. of demethylchlortetracycline hydrochloride, the latter being changed to 150 mg. capsules after the first few weeks. These antibiotics were used in the usual manner for the treatment of any infections in which they were considered to be indicated, but they were prescribed in terms of the same number of capsules rather than in terms of the same number of milligrams. Thus, during the first few weeks, the dose of demethylchlortetracycline was one-half that of tetracycline and subsequently about 60 per cent as much demethylchlortetracycline as tetracycline was given. For severe infections, two capsules were given every six hours but for mild infections or after defer-

TABLE II

Comparative Activity of 4 Tetracycline Analogues Against 733 Strains of Various Organisms

	No. of strains	Oxytetracycline	Tetracycline	Chlortetracycline	Demethylchlortetracycline
<i>Most Active Analogue, %</i>					
Sensitive*	600	42	21	29	65
Resistant†	133	22	49	11	25
<i>Average Activity Rating‡</i>					
Sensitive	600	1.6	1.8	1.9	1.3
Resistant	133	2.2	1.5	2.1	1.9

* Minimum inhibitory concentration of most active analogue = ≤ 25 .

† Minimum inhibitory concentration of most active analogue = ≥ 50 .

‡ This rating is mean of rating of all strains; for each strain, based on twofold dilution tests on agar plates: 1 = most active; 2 = 2x minimum inhibitory concentration of most active; 3 = 4x minimum inhibitory concentration of most active; 4 = 8x minimum inhibitory concentration of most active; and so on.

vescence in the others one capsule is prescribed every six hours. The initial dose of two capsules was often prescribed when the smaller amounts were given.

A total of 80 patients have been treated with demethylchlortetracycline and a similar number with tetracycline.* Since a large variety of infections of different severity in patients with different underlying conditions were included, it is not possible here to present all of the clinical details nor would they be meaningful. Suffice it to say that the clinical results obtained with either agent were indistinguishable as far as could be observed both from the point of view of the effect on bacteria and on the fever and symptoms.

As to the comparative toxicity, only 7 patients experienced any observable untoward effects from demethylchlortetracycline; most of these 7 patients had only mild nausea, 2 had frequent soft, bulky, but formed stools. Only 1 patient had some regurgitation and moderately severe diarrhea during administration of demethylchlortetracycline; this patient was on tube feeding and *Staph. aureus* appeared in large numbers in the stools but the symptomatology and the staphylococci cleared after the antibiotic was withdrawn. One patient who was receiving barbiturates and syrup of hydriodic acid concomitantly experienced a rash that cleared after all medication was stopped. Of interest is the fact that in 3 patients who had slight to moderate nausea and vomiting and in 2 who were having moderate diarrhea when treatment with demethylchlortetracycline was started, these symptoms cleared during the treatment. On the other hand, 3 patients who were having moderate to severe nausea and vomiting, 1 who was also having diarrhea, and another who had fever and a rash during administration of tetracycline were changed to demethylchlortetracycline without affecting these symptoms, which cleared only after that latter antibiotic was also discontinued. Gastrointestinal symptoms including diarrhea were more than twice as frequent in patients treated with tetracycline than they were among those treated with demethylchlortetracycline. This may well have been related to the larger doses of tetracycline that were used. Somewhat more than one half of the patients on each analogue received two capsules every six hours at least at the start of therapy. A few patients were given two capsules of demethylchlortetracycline every 8 or 12 hours; none of these had any untoward effects.

SUMMARY AND CONCLUSIONS

From all of the observations that have been presented it seems justified to conclude that demethylchlortetracycline is a new and highly active member of the tetracycline group that is more stable than any of the earlier three analogues. It is at least as active in vitro as any of them and is probably more active than the others against a majority of "susceptible" pathogens. Demethylchlortetracycline given orally yields much greater antibacterial activity and this extends over a considerably longer period than equivalent amounts of the other three analogues with which it was compared. From the limited experience to date, demethylchlortetracy-

* Some of the capsules of tetracycline that were used in this comparison contained equal amounts of glucosamine hydrochloride and some contained citric acid, but no adjuvants were contained in the capsules of demethylchlortetracycline that were used therapeutically.

cline may be expected to produce comparable clinical effects in the treatment of susceptible bacterial infections with the use of appreciably smaller doses, or with doses of similar amounts given less frequently than with the other analogues. The smaller or less frequent doses may also be expected to produce untoward effects less frequently. However, more clinical experience is required to substantiate these preliminary impressions.

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The Sensitivity of Selected Strains of *Shigella*, *Salmonella*, and Enteropathogenic *Escherichia coli* to Demethylchlortetracycline and Tetracycline

JORGE OLARTE

Hospital Infantil de México, México, D. F.

In 1957 McCormick et al¹ isolated a new tetracycline antibiotic, demethylchlortetracycline, produced by a mutant of the strain *Streptomyces aureofaciens* Duggar. Kunin and Finland² and Hirsch and Finland³ found that demethylchlortetracycline produces higher and more sustained levels of antibacterial activity in the blood of human subjects than those obtained with tetracycline, chlortetracycline, or oxytetracycline. According to the report of Sweeney et al,⁴ demethylchlortetracycline is more stable and more active against certain microorganisms than the other tetracyclines currently in use.

The purpose of the present study was to compare the action of demethylchlortetracycline and tetracycline against some of the most frequent *Shigella*, *Salmonella*, and enteropathogenic *Escherichia coli* types found in children with diarrhea at this hospital. *Salmonella typhi* and *Salmonella paratyphi* A were excluded from the study. Since in recent years the emergence of resistant strains of enteropathogenic bacteria to tetracyclines has been observed,^{5,8} a number of cultures previously known to be resistant to these antibiotics were intentionally included in the study.

MATERIAL AND METHODS

Strains Tested. Forty strains of *Shigella* of miscellaneous types (*Shigella flexneri*, 34; *Shigella sonnei*, 3; *Shigella shigae*, 3); 29 strains of *Salmonella* (*Salmonella typhimurium*, 14; *Salmonella oranienburg*, 4; *Salmonella enteritidis*, 2; other types, 9); and 40 strains of *E. coli* (*E. coli* 0111:B4, 16; *E. coli* 0127:B8, 3; *E. coli* 0119:B14, 2; *E. coli* 086:B7, 3; other types, 16) were tested. Each strain was isolated from a different patient during the period from June, 1958, to June, 1959.

Antibiotics Tested. Freshly prepared solutions of demethylchlortetracycline hydrochloride (lot 45745-791, Lederle) and tetracycline hydrochloride (lot 7-9705, Lederle) were used.

Test for Sensitivity. Each strain was tested by the plate dilution method described elsewhere.⁹ Brain-heart infusion agar, 1.5 per cent (Difco), was used. The final concentrations of the antibiotics were 50, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.07, and 0.03 µg./ml. The final volume was 10 ml. of medium per plate.

Each agar plate was inoculated with a three hour broth culture using sterile swabs for streaking. This seed culture was prepared by inoculating 10 ml. of broth with 0.1 ml. of a 20 hour culture.

The end point was taken as the minimal antibiotic concentration producing inhibition for 20 hours when incubated at 37 C. Consideration was also given to differences in the degree of inhibition observed in the streaks, from confluent to scattered (1 to 20 colonies in the streak) bacterial growth.

TABLE I

Comparison of the Activity of Demethylchlortetracycline and Tetracycline
on 109 Selected Strains of Enteropathogenic Bacteria

Bacteria	No. of strains tested	Strains resistant to both antibiotics*		Strains sensitive to both antibiotics at equal concentration†		Strains showing higher sensitivity to demethylchlortetracycline‡		Strains showing higher sensitivity to tetracycline‡	
		No.	%	No.	%	No.	%	No.	%
<i>Shigella</i>	40	24	60	1	3	15	37	0	
<i>Salmonella</i>	29	10	34	8	28	9	31	2	7
<i>E. coli</i>	40	17	42	7	18	4	10	12	30
Total	109	51	47	16	14	28	26	14	13

* Strains growing in 10 µg./ml. or more were regarded as resistant.

† All sensitive strains were inhibited by concentrations ranging from 0.3 to 1.25 µg./ml.

‡ The differences observed in sensitivity were never higher than one dilution, or those from confluent to scattered bacterial growth.

RESULTS

The results are shown in table I.

Twenty-four (60 per cent) *Shigella*, 10 (34 per cent) *Salmonella*, and 17 (42 per cent) *E. coli* strains were resistant to both antibiotics (demethylchlortetracycline and tetracycline) at concentrations of 10 µg./ml. or more.

One (3 per cent) *Shigella*, 8 (28 per cent) *Salmonella*, and 7 (18 per cent) *E. coli* cultures were inhibited by equal concentrations (ranging from 0.3 to 1.25 µg./ml.) of both antibiotics.

Fifteen (37 per cent) *Shigella*, 9 (31 per cent) *Salmonella*, and 4 (10 per cent) *E. coli* strains were inhibited by low concentrations (0.3 to 1.25 µg./ml.) of both antibiotics, but showed a higher sensitivity to demethylchlortetracycline than to tetracycline. Nevertheless, the differences observed were not higher than one dilution, or those from confluent bacterial growth for tetracycline and scattered bacterial growth for demethylchlortetracycline, at the same concentration. The opposite was true with 2 (7 per cent) *Salmonella* and 12 (30 per cent) *E. coli* cultures in which a slightly higher activity for tetracycline than that for demethylchlortetracycline was also observed at low concentrations.

SUMMARY AND CONCLUSIONS

A comparison was made of the activity of demethylchlortetracycline and tetracycline against 40 strains of *Shigella*, 29 strains of *Salmonella* (other than *Sal. typhi* and *Sal. paratyphi* A), and 40 strains of enteropathogenic *E. coli*.

Both antibiotics showed a similar activity against most of the cultures tested. In some instances (*Shigella*, 37 per cent, *Salmonella*, 31 per cent, and *E. coli*, 10 per cent), a slightly higher activity was shown by demethylchlortetracycline than by tetracycline; in other strains (*Salmonella*, 7 per cent, and *E. coli*, 30 per cent), a higher activity was obtained for tetracycline than for demethylchlortetracycline. However, the differences observed were apparently too small to be significant.

ACKNOWLEDGMENTS

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The Distribution and Excretion of Four Tetracycline Analogues in Normal Young Men

CALVIN M. KUNIN,* A. C. DORNBUSH, AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass., and the Biochemistry Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Three tetracycline analogues, tetracycline, chlortetracycline, and oxytetracycline have been used extensively in man and are well established as effective antimicrobial agents. Recently, a fourth analogue, demethylchlortetracycline was described and has been introduced to clinical use. The present report presents a comparison of the distribution and excretion of the four analogues as observed in the same 4 healthy young men following single intravenous injections of each. In addition data are presented on the plasma protein binding of the four analogues and the biliary excretion of demethylchlortetracycline. Detailed accounts of these studies are presented elsewhere.¹⁻²

Following intravenous injection of the four analogues an initial rapid decline of serum concentrations representing distribution in various body compartments was noted. This was complete at two to three hours. The half life of active drug was calculated by means of the method of least squares from the second slower part of the disappearance curve. The longest half life was observed with demethylchlortetracycline (12.7 hours); the shortest with chlortetracycline (5.6 hours); while oxytetracycline and tetracycline were intermediate (9.2 and 8.5 hours respectively).

The relative volumes of distribution of the four analogues were tetracycline, 1.59; oxytetracycline, 1.89; chlortetracycline, 1.48; demethylchlortetracycline, 1.79 times body weight. These relatively large values are probably due, in large part, to sequestration and concentration of these drugs in the liver and reticulo-endothelial system.

The renal clearance of each tetracycline analogue expressed as percentage of a simultaneous endogenous creatinine clearance was tetracycline, 62 ± 8 ; oxytetracycline, 85 ± 14 ; chlortetracycline, 30 ± 8 ; and demethylchlortetracycline, 27 ± 8 . Thus, there are marked differences in the rate of renal clearance of the analogues, but all clearances were less than that of creatinine. Variations in the rate of urine flow from 2 to 14 ml./minute did not significantly alter the rate of clearance of any of the analogues.

The differences in the renal clearance rates noted among the four analogues can in part be explained by the extent to which each is bound to plasma proteins, as determined by equilibrium dialysis. In our experiments wide variations were encountered, but the mean percentages of bound drug are tetracycline, 24; oxytetracy-

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* Present address: University of Virginia Medical College, Charlottesville, Va.

cline, 20; chlortetracycline, 48; and demethylchlortetracycline, 41. These values were determined by bioassay. Wozniak,³ using radiolabeled tetracycline analogues, found the binding in human serum to be tetracycline, 31 ± 6 ; chlortetracycline, 64 ± 4 ; and demethylchlortetracycline, 51 ± 2 per cent. These protein binding data account for much of the differences in renal clearance rates noted among the analogues, suggesting that the major mechanism of renal clearance of these drugs is by simple glomerular filtration.

The mean 96 hour urinary recoveries of the four analogues are tetracycline, 60 per cent; oxytetracycline, 70 per cent; chlortetracycline, 18 per cent; and demethylchlortetracycline, 42 per cent of the administered dose.

The nonrenal removal rates of tetracycline, oxytetracycline, and demethylchlortetracycline were very similar and averaged 3 to 4 per cent per hour; chlortetracycline, however, was removed much more rapidly by nonrenal mechanisms (9.5 per cent per hour).

The excretion of demethylchlortetracycline into the bile was determined in 4 women with biliary fistulas. Demethylchlortetracycline was found in high concentration in the bile at levels from 2 to 32 times greater than that of the serum. Thus, the slow disappearance of demethylchlortetracycline from the blood cannot be explained on the basis of delayed biliary excretion.²

Of the four analogues therefore demethylchlortetracycline clearly produces the most sustained levels in the blood; oxytetracycline is the most rapidly and completely excreted into the urine; tetracycline produces slightly less sustained levels in the blood and slightly lower urinary recoveries than oxytetracycline, and chlortetracycline yields the least sustained levels in blood and the smallest recoveries in the urine. It must be emphasized, however, that these pharmacological properties can only be interpreted, from the therapeutic point of view, in relation to the individual antimicrobial activity of each analogue against specific pathogenic organisms.

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Absorption of Demethylchlortetracycline in Children: Some Preliminary Observations

SYDNEY ROSS, JOSE R. PUIG, AND EDMUND A. ZAREMBA

Research Foundation of Children's Hospital, Washington, D. C.

In 1957, McCormick et al¹ described a new tetracycline compound produced by a mutant of the original strain of *Streptomyces aureofaciens*, which Duggar had used to produce chlortetracycline. This new antibiotic, demethylchlortetracycline,* differs from chlortetracycline only by the absence of the methyl group at the 6 position of the basic molecule; otherwise the two drugs are identical in their chemical configuration.

Demethylchlortetracycline has been found in previous studies to possess several definite advantages over its tetracycline analogues. These would include the following: Demethylchlortetracycline is much more resistant to degradation by acid or alkali;² it is excreted only about 43 per cent as rapidly as tetracycline, with a resulting longer half life;³ and as a consequence of slower excretion, demethylchlortetracycline has been found to produce significantly higher and better-sustained levels of antibacterial activity in adults than any of its congeners.²⁻⁴

The purpose of the present investigation was to determine whether the results of demethylchlortetracycline absorption studies, all of which had been done in adults, applied equally well to children, since the latter may absorb and excrete antibiotics at a rate different from adults.

MATERIAL AND METHODS

Three different preparations of demethylchlortetracycline intended for pediatric use were employed in the initial part of this study; these included a pediatric drop form, a suspension, and a syrup. Each was administered as a single 5 mg./Kg. dose on a fasting stomach to a group of children ranging in age from 3 to 8 years and varying in weight from 15 to 25 Kg. Blood was drawn for assays at 1, 3, 6, 12, and 24 hour intervals.

In a second experiment, a crossover study was performed on 5 children ranging in age from 4 to 11 years. Children in this group received a single 5 mg./Kg. dose of demethylchlortetracycline suspension and tetracycline† in rotation while fasting. As before, blood samples were drawn for assay at 1, 3, 6, 12, and 24 hour intervals.

The serum levels were measured by an agar diffusion cup plate method employing *Bacillus cereus* as the assay test organism.‡ Antibiotic activity was calculated

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin.

† The trade name of Lederle Laboratories Division, American Cyanamid Co., for a mixture of tetracycline base and sodium hexametaphosphate is Achromycin V syrup.

‡ Assays were kindly performed by Dr. A. C. Dornbush, Lederle Laboratories Division, American Cyanamid Co.

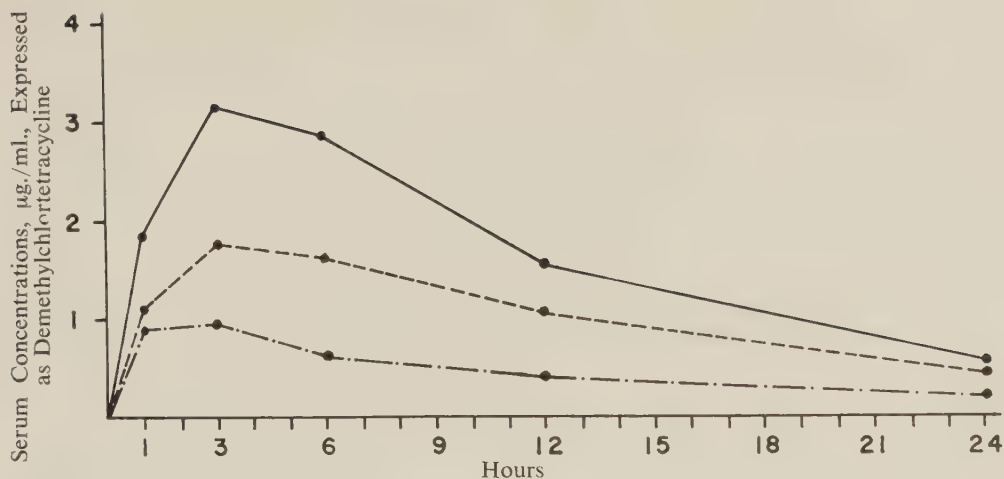


FIG. 1. Comparison between average serum concentrations obtained after administration of a single 5 mg./Kg. dose of three different pediatric preparations of demethylchlortetracycline. —•—, drop form; — — —, suspension; —•—, syrup.

both in terms of the antibiotic administered and in terms of tetracycline and demethylchlortetracycline equivalents.

RESULTS

Serum Demethylchlortetracycline Levels after Administration of Three Pediatric Dosage Forms. In figure 1 is presented a comparison of the average demethylchlortetracycline levels obtained after administration of a single 5 mg./Kg. dose of the pediatric drops to 5 children, of the suspension to 6 children, and of the syrup to 3 children. As will be noted, the highest levels were obtained with the pediatric drops. With the drops, an average peak level of 3.13 µg./ml. was achieved within three hours followed by a fairly constant therapeutic level during the next three hours (2.8 µg./ml.); at the end of 12 hours, the serum demethylchlortetracycline concentration had slowly tapered off to 1.5 µg./ml. and a trace level of 0.53 µg./ml. was still present after 24 hours.

When demethylchlortetracycline suspension was administered, the average levels

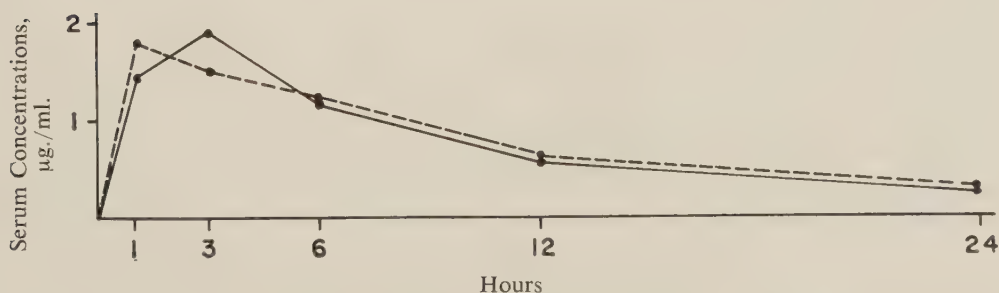


FIG. 2. Comparison between the average serum concentrations obtained in 5 children after they had received a single 5 mg./Kg. dose of demethylchlortetracycline and tetracycline in a crossover design experiment. (Values expressed in terms of the antibiotic administered.) —•—, demethylchlortetracycline suspension; — — —, tetracycline.

were 40 to 90 per cent lower than those obtained with a comparable dose of the pediatric drops. After a similar dose of the syrup, the levels were significantly lower than those obtained with either the drops or the suspension. The peak concentration at three hours was only 0.93 $\mu\text{g./ml.}$, as compared with 1.76 $\mu\text{g./ml.}$ with the suspension and 3.13 $\mu\text{g./ml.}$ with the drops. To a first approximation, the latter produced two and one half to four times greater demethylchlortetracycline serum concentrations than those obtained with the syrup, while the levels obtained with the suspension were intermediate between those with the other two preparations.

The reason for this significant disparity in serum concentration with these three demethylchlortetracycline pediatric preparations is not apparent at the present writing.

Serum Concentrations after Administration of Demethylchlortetracycline and Tetracycline in a Crossover Study. In figure 2 are shown the average serum antibiotic levels, expressed in terms of the antibiotic administered, after demethylchlortetracycline suspension and tetracycline were each given to a group of 5 children in a single 5 mg./Kg. dose in a crossover experiment. As will be observed, the one and three hour values for the drugs showed some minor variation, but thereafter, from 6 to 24 hours, the antibiotic activity for both drugs was quite similar.

When the demethylchlortetracycline levels are expressed as tetracycline equivalents, the comparison with the antibiotic activity expressed as demethylchlortetra-

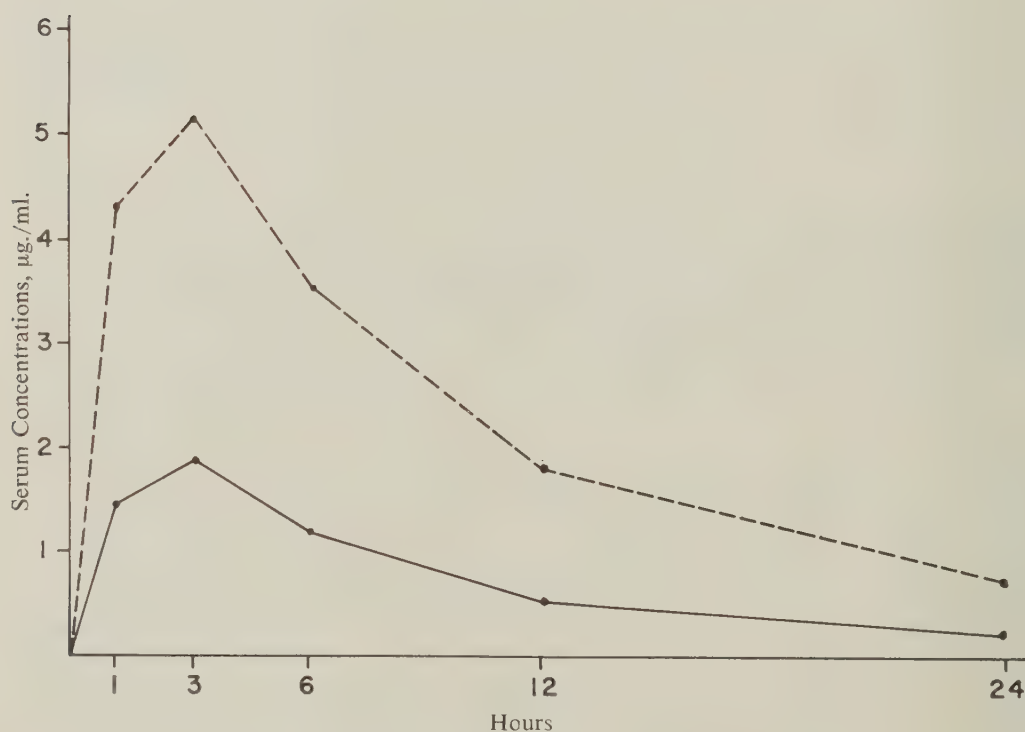


FIG. 3. Comparison of the average serum antibiotic activity, after administration of a single 5 mg./Kg. dose of demethylchlortetracycline, when the values are expressed as demethylchlortetracycline (—•—) and demethylchlortetracycline expressed as tetracycline equivalents (---•---).

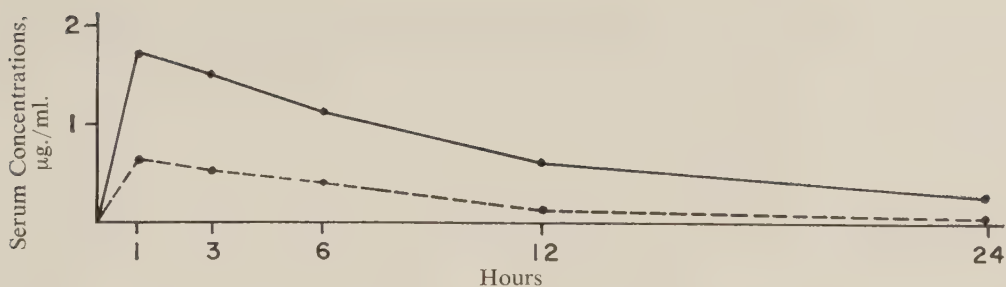


FIG. 4. Comparison of the average serum antibiotic activity, after administration of a single 5 mg./Kg. dose of tetracycline, when the values are expressed as tetracycline (—•—) and demethylchlortetracycline equivalents (---•---).

cycline is graphically illustrated in figure 3. Roughly, the antibiotic serum concentrations were three times greater when expressed as tetracycline equivalents than when expressed as demethylchlortetracycline activity.

Conversely, when the tetracycline levels obtained in the crossover experiment were expressed as demethylchlortetracycline equivalents (fig. 4), the latter were approximately one third as great as the serum concentrations obtained when expressed as tetracycline activity.

DISCUSSION

In the present study, serum concentrations expressed in terms of the antibiotic administered failed to show any significant difference between demethylchlortetracycline and tetracycline in a crossover experiment in 5 children receiving a single 5 mg./Kg. dose of each drug. However, single dose data may not be so definitive as those obtained after multiple dose studies, which would simulate actual clinical use of the drug. Kunin and Finland³ have pointed out that the advantage of demethylchlortetracycline over tetracycline in adults becomes more clearly evident during administration of repeated oral doses, due in large part to the slower renal clearance of demethylchlortetracycline with resulting higher and longer-sustained levels. Data obtained after multiple doses in infants and children would be necessary before any conclusions could be regarded as valid in the pediatric age group.

A point of considerable potential controversy would be the validity of representing antibiotic activity of these two antibiotic agents in terms of one standard of reference, namely, tetracycline. It has been shown that demethylchlortetracycline is approximately three times more active than tetracycline against the test organism *B. cereus* no. 5, which was used in the cup plate assay method. Hence, antibiotic activity predicated on the use of this test organism would inevitably show demethylchlortetracycline to have a considerable advantage over tetracycline when expressed as tetracycline equivalents. It is clear, however, that the superiority of demethylchlortetracycline over its analogues would be valid only if it could be demonstrated that the former is more active than its congeners against actual pathogens encountered clinically.

SUMMARY AND CONCLUSIONS

1. The new tetracycline antibiotic, demethylchlortetracycline, was administered

to a group of children in three pediatric dosage forms. Considerably higher serum levels were obtained after administration of comparable single 5 mg./Kg. doses of the drop preparation than those obtained with the syrup; the suspension yielded levels intermediate between those of the drops and the syrup. The reason for this disparity is not evident at the present writing.

2. In a crossover experiment, single 5 mg./Kg. doses of demethylchlortetracycline and tetracycline were found to produce similar antibiotic levels over a 24 hour period when antibiotic activity was expressed in terms of the antibiotic administered. When expressed in tetracycline equivalents, however, the serum concentrations produced by demethylchlortetracycline were approximately three times greater than those obtained with tetracycline, a reflection of the fact that the former drug is approximately three times more active than tetracycline against the test organism *B. cereus* no. 5, which was employed in the cup plate assay method.

3. The importance of demonstrating that demethylchlortetracycline is more active than its congeners, not only against *B. cereus* but also against pathogens encountered clinically, is emphasized.

ACKNOWLEDGMENTS

Demethylchlortetracycline was generously supplied by Dr. Stanton Hardy, Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York. We are indebted to Dr. William Sterling for his technical assistance.

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Demethylchlortetracycline: Serum Concentration Studies and Cerebrospinal Fluid Diffusion

WILLIAM P. BOGER AND JOHN J. GAVIN

Department of Research Therapeutics, Norristown State Hospital, Norristown, Pa.

An extensive comparative study of tetracycline preparations has been carried out in this laboratory and reported upon recently.¹ The results of this study showed clearly that tetracycline hydrochloride, alone or in the presence of an inert excipient, such as lactose, gave as high or higher serum concentrations following a given dose than did other tetracycline preparations that were combined with so-called potentiating adjuvants. Accordingly, it seemed appropriate to evaluate, in a comparative way, the new agent, demethylchlortetracycline.*

MATERIALS AND METHODS

All medications employed in the study were specifically assayed for their antibiotic activity and were found to contain within 10 per cent of the indicated contents. The demethylchlortetracycline capsules were from a lot especially prepared for investigational use. The two tetracycline hydrochloride preparations, combined with citric acid and with glucosamine, were hand-filled capsules from the same lots that were studied in previous work.¹

Medications were administered to patients in a fasting condition. All persons participating in the investigations specifically directed toward comparisons of preparations were healthy volunteers; the other individuals were hospitalized patients. Serum samples used for assay were obtained from clotted blood specimens, which were allowed to stand in the refrigerator overnight before separation. All assays were done by the standard cup-plate method using *Bacillus cereus* as the test organism.² As a control measure, all samples taken following the administration of a particular compound were assayed during the same test.

All assay results are reported in terms of concentrations of the specific antibiotic administered, as determined by using that same antibiotic in the preparation of the standard curve. Weighed samples of crystalline chlortetracycline, tetracycline hydrochloride, and demethylchlortetracycline were used to prepare the standard curves.

RESULTS

Comparison of Demethylchlortetracycline and Chlortetracycline. Since the molecular configurations of demethylchlortetracycline and chlortetracycline are so similar, differing only by a methyl group, it is natural to inquire what differences in properties the demethylation has conferred. Four individuals were studied in a

The studies reported here were made possible by a grant-in-aid made by Lederle Laboratories Division, American Cyanamid Co., to the Fund for Research Therapeutics.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin.

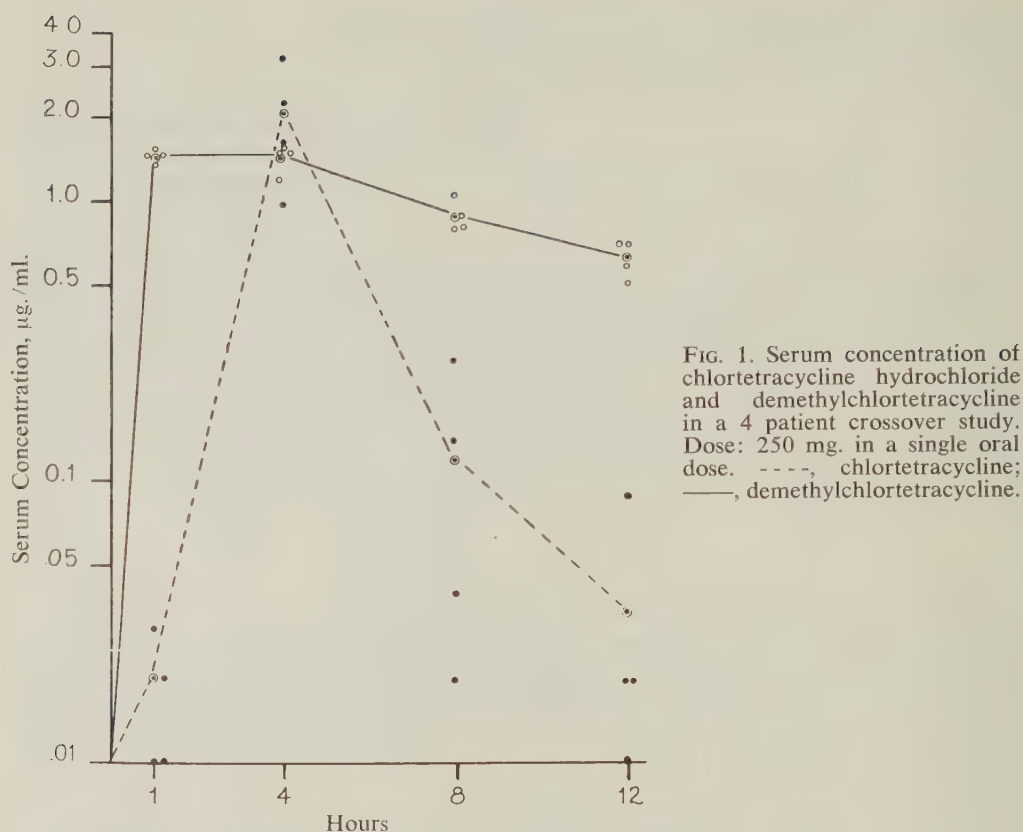


FIG. 1. Serum concentration of chlortetracycline hydrochloride and demethylchlortetracycline in a 4 patient crossover study. Dose: 250 mg. in a single oral dose. ----, chlortetracycline; —, demethylchlortetracycline.

crossover pattern, so that on each of the two days of study, 2 patients received chlortetracycline and 2 patients received demethylchlortetracycline. Single oral doses of 250 mg. were administered in a fasting condition, and 8 ounces of tap water were ingested. In figure 1 are presented the serum concentrations observed in this comparison.

Both drugs were promptly absorbed following oral ingestion, and during 12 hour periods of observation, the serum concentrations of demethylchlortetracycline declined much more slowly than did those following chlortetracycline. Based upon six hour observations following single oral doses, higher levels of chlortetracycline were observed. The suggestion has been made that over longer periods of observation the difference between chlortetracycline and demethylchlortetracycline would become more obvious.³ Our observations support and extend these prior findings.

Comparison of Demethylchlortetracycline and Tetracycline Hydrochloride with Citric Acid. Although it has been suggested that citric acid might serve as an enhancement agent to the absorption of tetracycline hydrochloride, our data fail to demonstrate any clear difference between tetracycline hydrochloride with or without citric acid.¹ At least the evidence seems clear that citric acid does not depress serum concentrations of tetracycline in the manner that has been demonstrated for calcium phosphate. Similar reasoning was used in the choice of tetracycline hydrochloride with citric acid for comparison with demethylchlortetracycline.⁴

In figure 2 are shown the results of a comparison in the same 6 patients studied in a crossover pattern. There is a slight average difference in favor of tetracycline hydrochloride with citric acid, without overlapping of values at one hour, but otherwise there appears to be no significant difference between the serum concentrations observed following the oral administration of single 250 mg. doses of tetracycline hydrochloride with citric acid and demethylchlortetracycline. Other workers have shown slightly greater difference in favor of tetracycline during the first three hours of observation after the single oral administration of 250 mg. doses.³ However, the two sets of data are in good agreement, and it is doubtful if the difference in the concentrations observed following the administration of single doses of these drugs is meaningful, particularly during the first six hours after ingestion.

Comparison of Demethylchlortetracycline and Tetracycline Hydrochloride with Glucosamine. Although glucosamine has been proposed as an adjuvant that enhances the serum concentrations of tetracycline, the information available is not convincing. Nevertheless, in the same manner as citric acid has been found to be free of chelating action and, hence, depressing effect upon serum concentrations, glucosamine has been found neither to inhibit nor to enhance tetracycline serum concentrations.¹

In figure 3 are presented the observations following a crossover comparison in 6 patients between tetracycline with glucosamine and demethylchlortetracycline. In somewhat the same fashion as is demonstrated in figure 2, the tetracycline with glucosamine gave an average value of serum concentrations slightly above those observed following the same 250 mg. dose of demethylchlortetracycline. The largest difference is observed in the first four hours after ingestion of the drugs. At 12 hours the crossing of the average lines suggests, as has been established by others,^{4,5} that demethylchlortetracycline is excreted more slowly than is tetracycline.

Repeated Doses of Demethylchlortetracycline and Accumulation. The demonstrated slower excretion of demethylchlortetracycline³⁻⁵ and the calculation that tetracycline is cleared by the kidney about 2.3 times faster than demethylchlortetra-

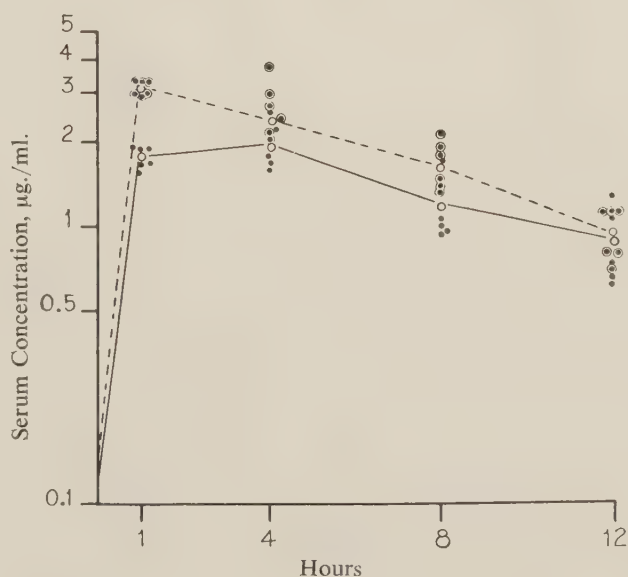


FIG. 2. Serum concentrations of tetracycline hydrochloride with citric acid and demethylchlortetracycline in a 6 patient crossover study. Dose: 250 mg. in a single oral dose. *—*—* tetracycline plus citric acid; •—•—• demethylchlortetracycline.

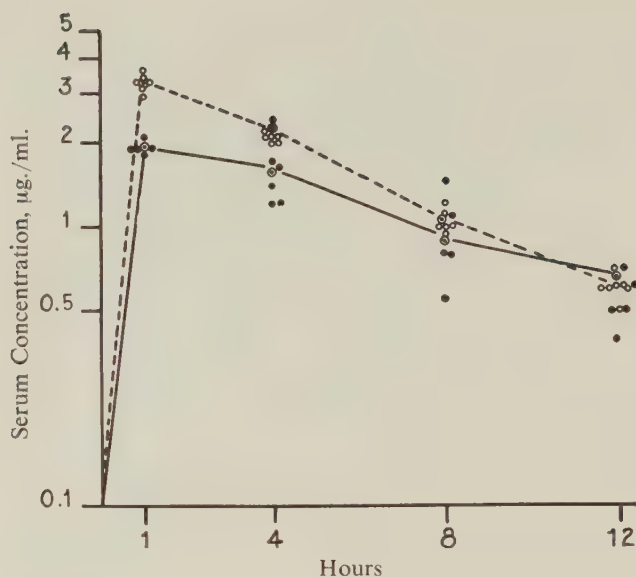


FIG. 3. Serum concentrations of tetracycline with glucosamine and demethylchlortetracycline in a 6 patient crossover study. Dose: 250 mg. in a single oral dose. ····· tetracycline plus glucosamine; —··— demethylchlortetracycline.

cycline⁴ suggest that the repeated administration of demethylchlortetracycline would result in a certain degree of accumulation in the circulation. To test this hypothesis, 9 individuals were given 125 mg. four times a day, and this schedule of treatment was continued for five days. On each day of medication, serum samples were obtained at one and four hours after the ingestion of the 11 a.m. dose. In figure 4 are presented the individual and average data from 5 women and 4 men. It is of interest to observe that as a group the women gave higher values than those seen in the men. This difference is a direct reflection of the lesser body weight of the women and, in consequence, a larger ingested dose on the basis of mg./Kg. Individually and compositely, there was a slight but definite rise in the serum con-

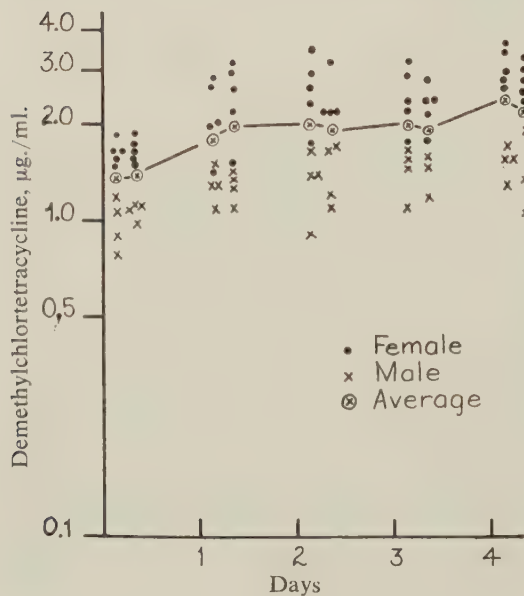


FIG. 4. Serum concentration of demethylchlortetracycline in 9 patients during multiple dose therapy, 125 mg. four times a day for five days.

centrations over this period of continuous therapy, so that the average values at the end of the fifth day of treatment were approximately twice those on the first day of treatment. This accumulation of the drug in the circulation is probably due to the slow rate of excretion of this antibiotic substance. The possibility of slower metabolism, greater protein binding, renal tubular reabsorption, and other considerations remain to be resolved.

Demethylchlortetracycline and Its Diffusion into the Cerebrospinal Fluid. A group of 32 patients, all of whom were scheduled for diagnostic lumbar punctures, were premedicated with demethylchlortetracycline 6, 8, and 12 hours prior to the performance of the test. At the same time that spinal taps were performed, simultaneous samples of blood and spinal fluid were obtained on each of the 32 patients. The results of the observations are shown in figure 5, and it is clear that at a time when the serum concentrations are declining, the small but, nevertheless, assayable amounts of demethylchlortetracycline are increasing in the cerebrospinal fluid. It is of interest that all of the patients showed antibiotic activity in the spinal fluid.

Whereas the foregoing 32 patients received a single oral dose of 500 mg., a smaller group of 10 patients were given a single oral dose of 1 Gm. four hours before diagnostic spinal taps were done. In table I can be seen the serum concentrations and the corresponding cerebrospinal fluid concentrations in simultaneously obtained specimens from 8 of these patients. By comparison with the results following 500 mg. doses, when samples were obtained at 6, 8, and 12 hours after medication, these values at four hours following 1 Gm. doses are higher in both serum and cerebrospinal fluid. It is clear that significant quantities of the drug diffuse promptly through the uninflamed meninges of man.

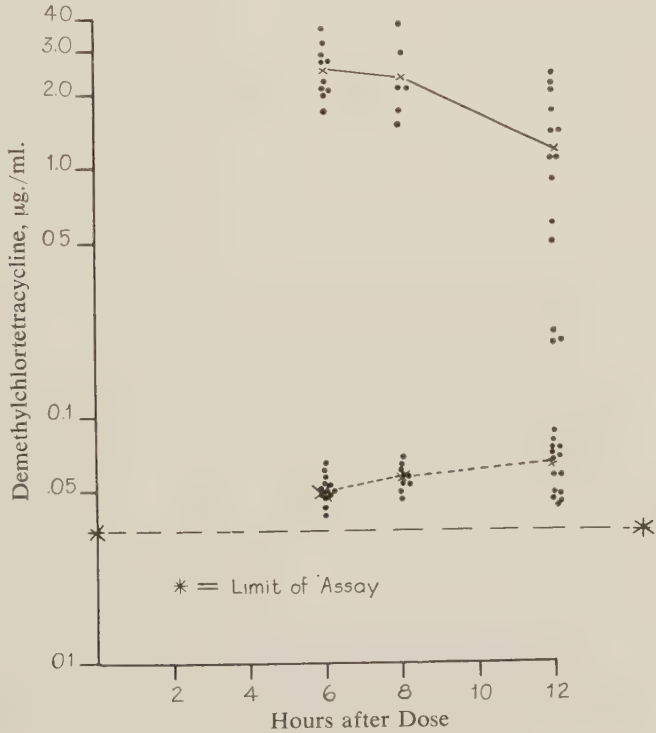


FIG. 5. Demethylchlortetracycline concentrations in serum (—) and spinal fluid (---) in paired specimens, after single oral dose of 500 mg. (32 patients).

TABLE I

*Paired Serum and Cerebrospinal Fluid Concentrations of Demethylchlortetracycline
in 8 Patients 4 Hours after Single 1 Gm. Oral Dose*

Patient	Age, yr.	Sex	Serum conc., μg./ml.	Cerebrospinal fluid conc., μg./ml.
L.R.	23	M	1.7	0.2
J.C.	29	M	2.5	0.17
G.E.	26	M	2.3	—
L.M.	22	M	1.7	0.17
J.N.	29	M	3.3	0.2
S.F.	35	M	2.7	0.21
P.H.*	46	F	3.3	0.2
S.N.*	38	F	5.7	—
R.P.*	26	F	3.9	0.21
U.W.*	54	F	7.5	0.17

* Patient showed nausea and vomiting.

All 4 of the women to whom 1 Gm. was administered orally had immediate gastrointestinal irritation. The fact that only the women became nauseated and vomited may be interpreted as a result of "overdosage," in light of the observations presented in figure 4 that indicated a tendency of the women to show higher serum concentrations on the same dose than did the men.

The over-all experience in 82 patients who received the drug showed a minimum of side effects, in contrast to the gastrointestinal irritation that was observed after the administration of a single 1 Gm. oral dose to a few underweight women. When 0.5 to 1 Gm. was administered daily in divided doses, only 2 of 82 patients showed any tendency toward nausea. Both of these patients were elderly and suffered from chronic urinary tract infections, along with a sufficient degree of renal impairment to have produced elevation of the nonprotein nitrogen in the blood.

DISCUSSION

In previous publications,^{3,5} serum concentrations of demethylchlortetracycline have been calculated in terms of "tetracycline activity." The justification for so doing is the demonstration that at the same μg./ml. concentration, demethylchlortetracycline in the standard cup-plate diffusion method gives a considerably larger zone of inhibition than does tetracycline. It might be postulated that this difference was based upon a difference in diffusion properties of the two compounds in solid medium, but it has been demonstrated by others that microgram for microgram, demethylchlortetracycline exerts a greater activity against a variety of organisms in liquid medium.^{4,5} Further, it has been shown that the sera of patients, to whom demethylchlortetracycline and tetracycline have been given in equal dosage, can be diluted to a greater extent following demethylchlortetracycline than after tetracycline and still show antibacterial effects.⁴

It is certainly true that in evaluating the extent to which a given antibiotic medication delivers therapy to the patient, the concentration of that substance in μg./ml.

per se is of little importance, whereas the amount of antibacterial activity represented by that number of micrograms is all important. The antibacterial activities of chlortetracycline, demethylchlortetracycline, and tetracycline, calculated as "tetracycline activity" on a microgram for microgram basis, show chlortetracycline to have the greatest activity.³ On the other hand, in comparing serum concentrations resulting from the same sized, single oral doses, those following chlortetracycline decline most rapidly. Accordingly, it rests upon clinical experience to establish whether the therapeutic value of chlortetracycline with a greater "tetracycline activity" and a more rapid excretion rate will be equalled or exceeded by demethylchlortetracycline, with its lesser "tetracycline activity" but more prolonged serum concentrations. This consideration may be more or less academic, inasmuch as chlortetracycline has been replaced (at least in the United States) almost entirely by tetracycline.

If the greater antibacterial effect of demethylchlortetracycline, as compared with tetracycline, is established at the clinical level, then it would appear that smaller doses can be administered with equal therapeutic effect. Since demethylchlortetracycline is so much more slowly excreted from the body than is tetracycline, it may be anticipated that there will be an increase in antibacterial effect as treatment is continued and that, upon discontinuance of the treatment, there will be residual circulating drug in the system for some 72 to 96 hours. A smaller daily dose, less gastrointestinal disturbance with this smaller dose, and more prolonged antibacterial effectiveness may prove to be advantages of demethylchlortetracycline over tetracycline hydrochloride. Additional clinical work is required to establish these points.

CONCLUSIONS

Single oral 250 mg. doses of demethylchlortetracycline gave equally high but more prolonged serum concentrations than did chlortetracycline. The $\mu\text{g./ml.}$ serum concentrations of demethylchlortetracycline are comparable to those following tetracycline hydrochloride in combination with citric acid or glucosamine. When the drug is administered for a period of five days, there is a slight, but definite accumulation in the serum due to a slower rate of renal excretion of demethylchlortetracycline than for either chlortetracycline or tetracycline.

Small, but measurable quantities of demethylchlortetracycline diffuse through uninflamed meninges into the spinal fluid following single oral doses of 500 or 1000 mg. doses.

Demethylchlortetracycline administered in a divided daily dose of 0.5 to 1 Gm./day to 82 patients for a variety of clinical conditions was well tolerated and effective. This new tetracycline derivative is characterized by rapid gastrointestinal absorption, slow renal excretion, diffusibility through uninflamed meninges, and greater in vitro antibacterial activity than tetracycline.

ACKNOWLEDGMENT

The authors are indebted to Mr. Vincent Cassella for technical assistance, and to Lederle Laboratories Division, Pearl River, N. Y., for the generous supplies of demethylchlortetracycline.

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Clinical and Laboratory Evaluation of Demethylchlortetracycline

JOHN P. VINEYARD, JANET HOGAN, AND JAY P. SANFORD

*Department of Internal Medicine, The University of Texas Southwestern
Medical School, Dallas, Texas*

Demethylchlortetracycline, a new tetracycline compound closely related to the existing tetracyclines, is produced by a mutant of the strain *Streptomyces aureofaciens* Duggar.¹ Demethylchlortetracycline produced greater and more sustained levels of antibacterial activity in the serum of normal subjects than the levels observed following comparable amounts of tetracycline, chlortetracycline, or oxytetracycline.²⁻⁴

In view of these observations, further laboratory studies and clinical trials in patients hospitalized with a variety of infections were undertaken.

METHODS

The group of patients selected for study consisted of individuals hospitalized on the medical and obstetrical-gynecological services. Demethylchlortetracycline capsules (150 mg.) were administered in place of tetracycline capsules (250 mg.) when the latter were ordered on these services. Thus, the dosage employed varied from 600 to 1200 mg. daily to correspond to the common daily usage of 1.0 to 2.0 Gm. of tetracycline. The patients were seen by us daily in order to evaluate therapy and ascertain the occurrence of side effects without asking leading questions that might suggest untoward reactions to the patients. Specimens were obtained for bacteriological examination prior to the institution of antibacterial therapy in most patients, except those with pelvic inflammatory disease. Standard clinical bacteriological methods were employed. All urine specimens had quantitative bacterial counts performed according to previously described methods.⁵ Follow-up cultures were obtained when clinically possible, specifically in patients with pyelonephritis. Clinical evaluation was based upon subjective and objective improvement in the patient, decrease in fever, return of leukocyte counts to normal, and, when applicable, resolution of radiological lesions and normalization of bacteriological cultures.

In vitro studies were performed on 964 bacterial strains, which were considered to be pathogens, freshly isolated from specimens submitted to the bacteriological laboratory during the period of July through September, 1959. The following tetracycline formulations were compared simultaneously: demethylchlortetracycline hydrochloride, lot 7-9454; tetracycline hydrochloride, lot 45745-196; chlortetracycline hydrochloride, lot 45744-191; and oxytetracycline hydrochloride, lot 92409. Each of these was assayed for potency. The in vitro sensitivities were carried out using the agar-dilution plate technique. Twofold dilutions of antibiotic, ranging from 0.312 to 20 µg./ml., were made in Trypticase soy agar, and the Petri plates

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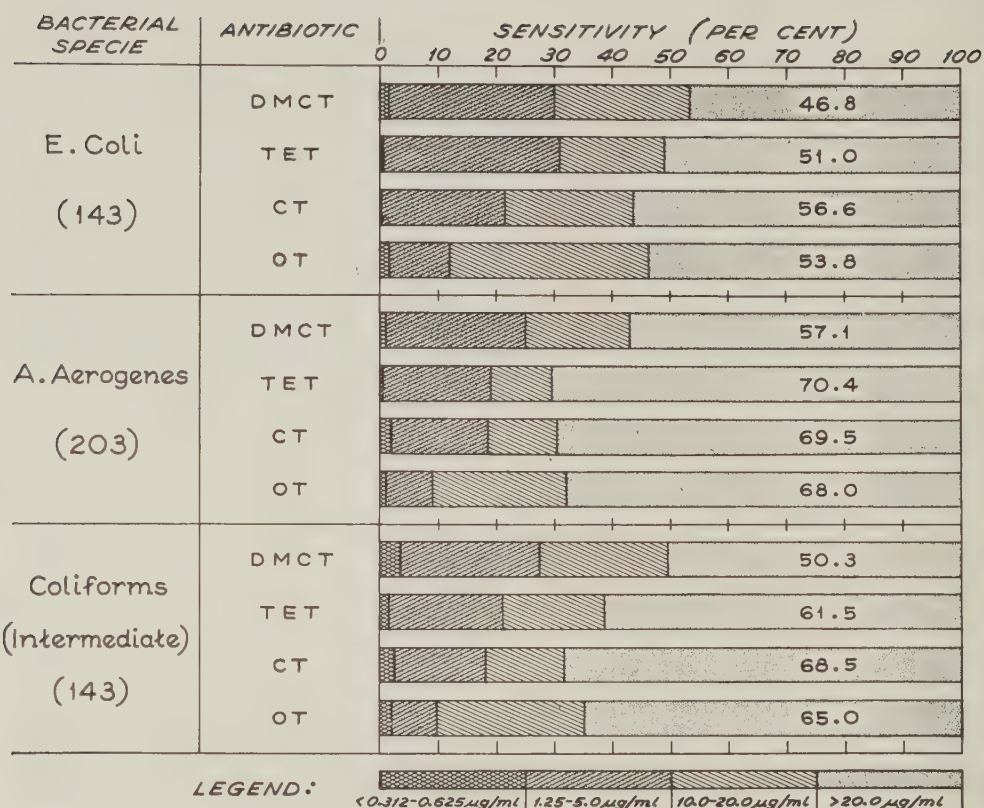


FIG. 1. Comparative sensitivity of bacteria to tetracycline analogues. DMCT = demethylchlortetracycline; TET = tetracycline; CT = chlortetracycline; OT = oxytetracycline.

were stored at 4 C. for a maximum period of seven days. These plates were marked with a grid, and each square (36) was inoculated with a cotton swab moistened in an overnight Trypticase soy broth culture of the organism to be tested. The sensitivity tests were incubated 18 to 20 hours at 37 C. and read macroscopically to the minimum concentration of antibiotic that completely inhibited growth.

RESULTS

A comparison of the in vitro susceptibility of various bacterial species to demethylchlortetracycline, tetracycline, chlortetracycline, and oxytetracycline is presented in figures 1 to 3.

The major sources from which these organisms were isolated is as follows: *Escherichia coli*, 77 per cent from urine cultures; *Aerobacter aerogenes*, 67 per cent from urine cultures; and coagulase-positive staphylococci, 36 per cent from wounds, 13 per cent from sputum, and 11 per cent from breast abscesses.

The group designated as "intermediate coliforms" includes the following strains: *Escherichia freundii* 11, *Escherichia intermedium* 43, *Paracolobactrum* species 85, and *Alcaligenes faecalis* 4. These organisms were grouped together to facilitate presentation, though on analysis of the individual species the sensitivity patterns are quite similar. Likewise, the strains belonging to the *Proteus* species were studied

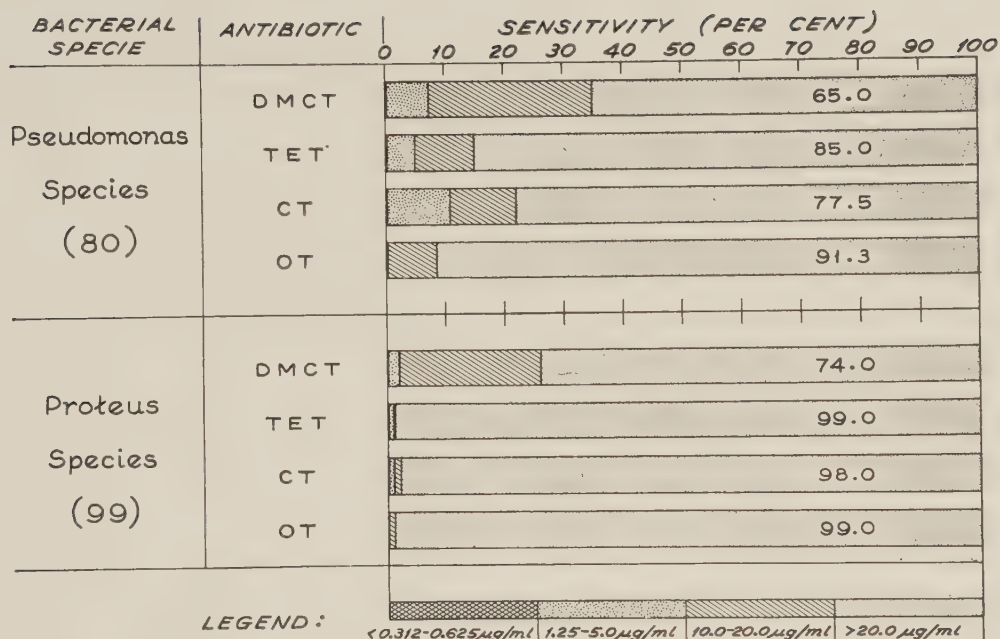


FIG. 2. Comparative sensitivity of bacteria to tetracycline analogues. DMCT = demethyl-chlortetracycline; TET = tetracycline; CT = chlortetracycline; OT = oxytetracycline.

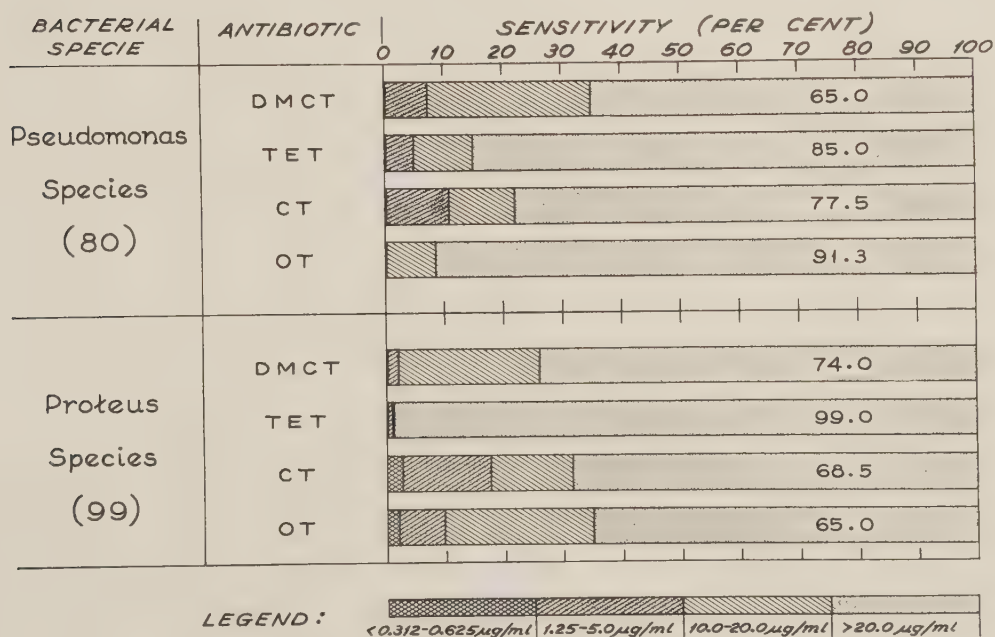


FIG. 3. Comparative sensitivity of bacteria to tetracycline analogues. DMCT = demethyl-chlortetracycline; TET = tetracycline; CT = chlortetracycline; OT = oxytetracycline.

individually: *Proteus vulgaris* 58, *Proteus mirabilis* 26, *Proteus rettgeri* 13, and *Proteus morgani* 2. The proportion of strains in each of these species of *Proteus* sensitive to 20 µg./ml. or less of demethylchlortetracycline ranged from 11 to 31 per cent.

It should be noted that the percentage of organisms sensitive to 20 µg./ml. or less of demethylchlortetracycline exceeded that sensitive to the other tetracyclines, with the exception of strains of coagulase-positive staphylococci and the percentage of enterococci sensitive to chlortetracycline. The extreme sensitivity (less than 0.312 µg./ml.) of 3.3 to 12.4 per cent of the coagulase-positive staphylococci was of interest. Analysis of culture sources showed that 65 per cent of these sensitive strains were isolated from wounds, furuncles, or blood cultures, which represent nonhospital-acquired staphylococcal infections.

Three strains of *Salmonella typhosa* were studied and were found susceptible to 1.25 to 5.0 µg./ml. of demethylchlortetracycline. All were sensitive to 20 µg./ml. or less of the other tetracyclines, except one that was resistant to this concentration of oxytetracycline.

The in vitro efficacy of demethylchlortetracycline, when compared to the other three tetracyclines, is summarized in table I.

Fifty-eight patients were treated with demethylchlortetracycline as either the sole antimicrobial agent or in combination with other antibiotics (tables II and III). The dosage schedules employed were either 150 or 300 mg. at six hour intervals. These schedules were tabulated separately to ascertain differences in either effectiveness or side effects.

The patients included in the groups designated "pneumonitis" consisted predominantly of individuals with chronic bronchopulmonary disorders, such as emphysema with superimposed acute bacterial infection and bronchopneumonia following aspiration of oral or gastric contents. There were no patients in this group with pneumococcal lobar pneumonia, as these studies were performed from mid-July through September, 1959. Sputum cultures obtained prior to institution of therapy showed a wide variety of microorganisms, many of which may represent the normal flora of the upper respiratory tract. Evaluation of an antimicrobial agent

TABLE I
Comparison of Demethylchlortetracycline with Other Tetracyclines

Organism	No. of strains	Per cent sensitive demethylchlortetracycline only	Per cent resistant strains sensitive to other tetracyclines		
			Tetra-cycline	Chlortetra-cycline	Oxytetra-cycline
<i>E. coli</i>	143	5.6	7.0	5.6	6.3
<i>A. aerogenes</i>	203	21.2	1.0	2.5	3.0
Intermediate coliforms	143	11.2	4.9	1.4	4.9
<i>Pseudomonas aeruginosa</i>	80	17.5	2.5	6.2	0
Staphylococci, coagulase positive	273	1.5	1.8	4.4	1.8
Enterococci	20	0	0	0	0
<i>Proteus</i> species	99	24.0	0	0	0

TABLE II

Summary of Clinical Observations with Demethylchlortetracycline as Single Antimicrobial Agent

Major disease	No. patients	Dosage, mg. daily	Duration of treatment, days		Results		
			Mean	Range	Improved	Not improved	Side effects
Pneumonitis	4	1200	16.0	9-32	4	0	0
	3	600	9.7	8-12	2	1	0
Pyelonephritis	3	600	7.0	6-9	3	0	0
Pelvic inflammatory disease	1	1200	7.0		1	0	1
	4	600	7.5	6-10	4	0	0
Misc. infections	1	1200	8.0		1	0	0
	4	600	6.8	3-9	4	0	0
Total	20				19	1	1
Average			9.3	3-32			

in this type of patient is exceedingly difficult, as the natural course of these types of infections is not predictable. We gained the clinical impression, from review of their courses of hospitalization, that their responses were satisfactory and comparable to those anticipated with tetracycline or the other tetracycline analogues.

The efficacy of an antibiotic can be more objectively evaluated in patients with pyelonephritis, since urine cultures can be followed. The observations on 13 patients with pyelonephritis are presented in table IV. The initial urine cultures obtained from these patients all contained greater than 500,000 organisms/ml. of urine, except patient A. Lo. These cultures were thought to represent infection, not contaminants, in all instances. Most of the patients had acute infections rather than chronic infections, which had failed to respond to numerous other agents. This is substantiated by the demonstration that 13 of the 16 bacterial strains cultured were

TABLE III

Summary of Clinical Observations with Demethylchlortetracycline in Combination with Other Antimicrobial Agents

Major disease	No. pt.	Other antibiotics	Dosage demethylchlortetracycline, mg. daily	Duration of treatment, days		Results		
				Mean	Range	Improved	Not improved	Side effects
Pneumonitis	8	Penicillin	1200	20.6	6-58	8	0	1
	11	Penicillin	600	12.5	6-29	10	1	1
	1	Streptomycin	1200	9.		1	0	1
	1	Erythromycin	600	12.		1	0	0
Pyelonephritis	1	Streptomycin	1200	9.		1	0	0
	8	Streptomycin	600	8.4	3-11	6	2	2
	1	Kanamycin plus polymyxin B	600	13		1	0	1
Misc. infections	2	Penicillin	1200	9.5	9-10	2	0	0
	4	Penicillin	600	9.0	5-13	4	0	0
	1	Streptomycin	600	4.0		1	0	0
Total	38					35	3	6
Average				12.2	3-58			

TABLE IV

Clinical Observations in Patients with Pyelonephritis Treated with Demethylchlortetracycline

Patient	Additional antibiotics given	Duration treatment, days	Initial organism	Sensitivity to <20 µg./ml. demethylchlortetracycline	Result*
M. T.	Streptomycin	11	Paracolon	+	4+
C. W.	Streptomycin	10	<i>A. aerogenes</i>	+	4+
A. St.	Kanamycin,		Paracolon	Not done	4+
	polymyxin B	13	<i>E. coli</i>	+	4+
H. D.	Streptomycin	10	<i>E. coli</i>	+	4+
O. H.	Streptomycin	10	Paracolon	+	4+
			<i>A. aerogenes</i>	+	4+
A. Sm.		9	Enterococcus	+	4+
E. L.		6	<i>E. coli</i>	+	4+
A. Lo.		6	Culture negative		2+
M. A.	Streptomycin	14	<i>E. coli</i>	+	4+
M. W.	Streptomycin	9	Paracolon	+	4+
B. G.	Penicillin		Paracolon	+	2+
			Enterococcus	—	
			<i>A. aerogenes</i>	—	
A. La.	Streptomycin	5	<i>E. coli</i>	+	2+
			Paracolon	+	
A. Sv.	Streptomycin	4	<i>A. aerogenes</i>	—	0

* Results expressed as: 4+ = sterile culture following therapy; 2+ = symptomatic improvement, culture remained positive or not done; 0 = no improvement.

sensitive to demethylchlortetracycline. Therapy was bacteriologically successful in three fourths of this group of patients, which is in accord with that noted by others in acute pyelonephritis.^{6,7}

Side effects were noted in 7 patients; 3 had nausea and 4 had nausea and vomiting. The frequency was higher on the 1200 mg. daily dosage (18 per cent) than on 600 mg. daily (10 per cent). The side effects did not necessitate the discontinuance of medications. The drug was well tolerated by the other patients.

DISCUSSION

The new tetracycline compound, demethylchlortetracycline, has an antibacterial spectrum that approximates that of the other three tetracyclines—tetracycline, chlortetracycline, and oxytetracycline. While cross resistance between the tetracycline compounds is usual, it is not absolute, and each antibiotic must be judged on its own merits. In simultaneous in vitro sensitivity studies, demethylchlortetracycline was effective in vitro against more strains of the gram-negative bacteria commonly associated with urogenital infections than were the other tetracyclines. The greater susceptibility to demethylchlortetracycline was most marked with strains of *A. aerogenes*, *Proteus* species, and *Pseudomonas aeruginosa*. It remains to be seen whether a discrepancy between in vitro susceptibility and in vivo response, similar to that occurring with oxytetracycline, will be noted in infections with *Ps. aeruginosa* treated with demethylchlortetracycline.⁸

Thus, on the basis of in vitro studies, demethylchlortetracycline would appear

to be the tetracycline of choice in many of the infections caused by species of gram-negative bacteria that tend to be resistant to the other tetracyclines.

The group of 58 patients treated with demethylchlortetracycline is difficult to evaluate from the standpoint of an objective comparison with the other tetracyclines. Two thirds of the patients had infections that were considered to warrant combined antibiotic therapy, and they received one of the bactericidal antibiotics in combination with demethylchlortetracycline. Penicillin and demethylchlortetracycline were usually administered to those patients with mixed type pneumonitis, and streptomycin and demethylchlortetracycline to those patients with pyelonephritis. However, the results of therapy in those patients with pyelonephritis, in whom the infection was associated with organisms that were sensitive *in vitro*, were very favorable. Side effects were noted in 7 patients (12 per cent). These were very mild.

The usefulness of any new antimicrobial agent must be considered in the light of other available agents. Are there advantages to the use of demethylchlortetracycline? The higher serum levels and greater duration of activity reported by Kunin and Finland² and Hirsch and Finland⁴ suggest that demethylchlortetracycline is a useful addition to the antibiotic armamentarium.

The enhanced level of antibacterial activity in the serum of normal volunteers, demonstrated in previous studies,^{2,4} was considered to be the result of a greater sensitivity of the assay organisms to demethylchlortetracycline than to the analogues, tetracycline, chlortetracycline, and oxytetracycline. As stated by Hirsch and Finland,⁴ "comparisons based only on concentrations of the antibiotics administered, . . . do not compare their relative antibacterial activity, which is the purpose for which they are used therapeutically; such comparisons may therefore be deceptive." Likewise, it should be emphasized that comparisons based upon relative antibacterial activity may be deceptive if the assay organisms employed are always more sensitive to antibiotic A than antibiotic B. If in the therapy of infections, organisms were encountered that were more sensitive to antibiotic B than antibiotic A, the relative antibacterial activity of antibiotic B would exceed that of antibiotic A. It would seem to be of more value to compare antibiotic activity on the combined basis of concentration and sensitivity of the specific organism in question.

Using this approach, the serum concentrations in $\mu\text{g./ml.}$ of tetracycline expressed as tetracycline and demethylchlortetracycline expressed as demethylchlortetracycline are comparable; however, since the assay organism (*Bacillus cereus* 5) is more sensitive to demethylchlortetracycline, greater relative antibacterial activity will be achieved. The *in vitro* sensitivities then are of considerable importance in selection of the agent. If the organism to be eradicated were more sensitive *in vitro* to tetracycline than to demethylchlortetracycline, then tetracycline would result in greater relative antibacterial activity. Thus, the major advantage of demethylchlortetracycline over the other analogues of tetracycline would be that a higher proportion of organisms are sensitive to this compound.

CONCLUSIONS

1. The new tetracycline compound, demethylchlortetracycline, demonstrates a spectrum of *in vitro* activity similar to tetracycline, chlortetracycline, and oxytetra-

cycline. It is effective in vitro against more strains of *Aerobacter aerogenes*, *Proteus* species, and *Pseudomonas aeruginosa* than are the other tetracycline analogues.

2. Clinical observations were carried out in 58 patients with a variety of infections. Significant improvement correlated reasonably well with in vitro predications in patients with pyelonephritis. Side effects were minimal and were observed in 7 persons. These were primarily nausea with or without vomiting.

3. Demethylchlortetracycline represents an important addition to the tetracycline analogues because of its greater antibacterial effectiveness against many bacteria and its lack of serious side effects.

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Demethylchlortetracycline: A Clinical and Laboratory Appraisal

DAVID M. PERRY, GEORGE A. HALL, AND WILLIAM M. M. KIRBY

University of Washington School of Medicine and the King County Hospital, Seattle, Wash.

Demethylchlortetracycline* was isolated by McCormick and co-workers in 1957.¹ This new compound was produced from a mutant strain of *Streptomyces aureofaciens*; it is structurally closely related to the known tetracyclines. It was found to be more stable with changes in pH than the other tetracyclines.

Sweeney et al² found that during the first six hours after the ingestion of equivalent doses, demethylchlortetracycline produced blood levels ($\mu\text{g./ml.}$) that were lower than levels produced by tetracycline. Kunin and Finland,³ in a multiple dose study, found that the average serum concentrations of demethylchlortetracycline and tetracycline were not significantly different four hours after the first dose, but that thereafter, levels of demethylchlortetracycline were higher than with tetracycline. When expressed in terms of serum antibacterial activity, the authors reported that demethylchlortetracycline, in single or repeated oral doses, produced much higher and better sustained levels of activity than corresponding doses of tetracycline. The difference between the serum concentrations and the serum activity was considered to result from the greater activity of demethylchlortetracycline against the assay organism and its longer half-life in the serum, as compared with tetracycline. In a later study, Hirsch and Finland⁴ found that demethylchlortetracycline also produced greater and better sustained serum antibacterial activity than equivalent doses of oxytetracycline and chlortetracycline.

This paper deals with laboratory and clinical studies of demethylchlortetracycline. The susceptibilities of various bacteria to tetracycline and demethylchlortetracycline were determined. Crossover studies were carried out in healthy volunteers, comparing antibacterial serum activity following a single dose of 0.3 Gm. of demethylchlortetracycline and 0.5 Gm. of tetracycline. These amounts were used in order to compare doses that might be used in clinical practice. In addition, 47 patients with respiratory and urinary tract infections were treated with demethylchlortetracycline, and the clinical response and tolerance to the medication were noted. In 13 patients, serum levels were determined after multiple doses of the new tetracycline compound.

MATERIALS AND METHODS

Susceptibility of Various Bacteria to Tetracycline and Demethylchlortetracycline. The organisms used in this study included pneumococci, beta-hemolytic streptococci, and coliform organisms, which were isolated from patients at the King County

This work was supported by a grant from Lederle Laboratories Division, American Cyanamid Co.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline hydrochloride is Declomycin. Capsules used throughout this study were kindly supplied by Dr. Stanton Hardy of Lederle Laboratories Division.

Hospital. Pneumococci were identified by their cultural characteristics and sensitivity to optochin, and group A hemolytic streptococci were identified by Lancefield grouping. The minimum inhibitory concentrations of tetracycline and demethylchlortetracycline were determined by a tube dilution method. Tubes containing 0.5 ml. of the various dilutions of each antibiotic were inoculated with 0.5 ml. of a 10^{-2} dilution of an 18 to 20 hour broth culture of each organism. The final dilutions of the antibiotics were 8.0, 4.0, 2.0, 0.5, 0.25, 0.1, and 0.05 $\mu\text{g./ml.}$ The minimum inhibitory concentration for each antibiotic was determined macroscopically after overnight incubation at 37 C. Tryptose phosphate broth (Difco) was used for coliform organisms and staphylococci, and tryptose phosphate broth with 3 per cent blood was used for pneumococci and streptococci.

Measurement of Serum Levels. A comparison of the serum antistreptococcal activity was carried out in 10 subjects after the ingestion of 0.3 Gm. of demethylchlortetracycline hydrochloride and 0.5 Gm. of tetracycline hydrochloride. Four of the subjects received glucosamine-potentiated tetracycline,* and 6 subjects received tetracycline buffered with citric acid.† The subjects were normal young adults who ranged in age from 23 to 28 years and in weight from 124 to 170 pounds. The test antibiotic was ingested after an overnight fast of nine hours, with intervals of three or more days between doses. Blood specimens were drawn 2, 4, 6, 12, and 24 hours later. The serum was extracted and stored at -20°C. until the time of the assay. Serum levels were determined by a modification of the penicillin assay technique of Rammelkamp,⁵ as described by Ziegler and McGuire.⁶ The test organism used in the assays was susceptible to 0.05 $\mu\text{g.}$ of demethylchlortetracycline and to 0.1 $\mu\text{g.}$ of tetracycline, and these minimum inhibitory concentrations were confirmed simultaneously with each serum assay. The details of the method used in this laboratory are reported elsewhere.⁷

In order to compare the relative incidence and severity of gastrointestinal disturbances, each volunteer was asked before taking the drug to report any ill effects experienced in the ensuing 24 hours. The volunteers were not told which of the preparations they were taking.

Serum levels were also determined in patients with acute respiratory infections after multiple doses. Three of these patients received at least six 0.3 Gm. doses of demethylchlortetracycline at 12 hour intervals. Ten patients received at least 12 doses of 0.3 Gm. of demethylchlortetracycline at six hour intervals prior to the test. Blood samples were drawn at 2, 4, 6, and 12 hours after the 9 a.m. dose on the day of the test.

Clinical Trial. Forty-seven patients admitted to the King County Hospital were treated with demethylchlortetracycline. Thirty had acute bacterial pneumonia, 6 had acute bronchitis with fever and purulent sputum, and 4 had urinary tract infections. Seven of the patients had illnesses that did not turn out to be acute bacterial infections. There were 29 men and 18 women, and their ages ranged from 20 to 85 years. Eighteen patients received 0.3 Gm. of demethylchlortetracycline twice daily, and 29 patients received 0.3 Gm. four times daily (every six hours).

* The trade name of Chas. Pfizer & Co. for glucosamine-potentiated tetracycline is Cosa-Tetracycln.

† The trade name of Lederle Laboratories Division, American Cyanamid Co., for tetracycline hydrochloride buffered with citric acid is Achromycin V.

TABLE I

Tube Dilution Tests with Demethylchlortetracycline and Tetracycline Against 150 Strains of Recently Isolated Pathogenic Bacteria

Organism	Drug	No. strains	Minimum inhibitory concentration, $\mu\text{g./ml.}$									
			16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.1	0.05	
Staphylococci	Tetracycline	50				2	26	22				
	Demethylchlortetracycline						2	39	8	1		
Pneumococci	Tetracycline	25						5	4	10	6	
	Demethylchlortetracycline							4	3	7	11	
Streptococci (group A)	Tetracycline	35	4	5	1	0	3	5	6	8	3	
	Demethylchlortetracycline			1	6	2	2	3	3	10	8	
Coli-aerogenes organisms	Tetracycline	25										
	Demethylchlortetracycline		1	0	7	12	5					
				1	1	7	8	8				
Streptococci (other than group A)	Tetracycline	15										
	Demethylchlortetracycline							3	4	4	4	
								1	3	3	8	

The patients were seen daily to evaluate the clinical response and to note side effects.

RESULTS

Susceptibility of Bacteria to Demethylchlortetracycline. The minimum inhibitory concentrations of tetracycline and demethylchlortetracycline for 150 strains of bacteria are presented in table I. It can be seen that in each group of bacteria tested the strains tended to be inhibited by a lower concentration of demethylchlortetracycline than tetracycline. In most instances the difference in concentration was twofold. Five of the 25 coliform organisms and five of the 35 group A streptococci were four times more susceptible to demethylchlortetracycline. It is of interest that a few group A streptococci were relatively resistant to tetracycline.

Table II summarizes the comparative inhibitory concentrations of the two antibiotics. Of the 150 organisms tested, the minimum inhibitory concentrations were equal in 47 instances, and in 103 instances demethylchlortetracycline was more active. Seventy-four per cent of the staphylococci, 44 per cent of the pneumococci,

TABLE II

Comparison of in Vitro Activities of Demethylchlortetracycline and Tetracycline Against 150 Strains of Bacteria

Organisms tested	No. strains	MIC*		Demethylchlortetracycline more active than tetracycline (per cent)
		equal for both drugs		
Staphylococci	50	13	37	(74)
Pneumococci	25	14	11	(44)
Group A streptococci	35	7	28	(80)
Coli-aerogenes organisms	25	4	21	(84)
Streptococci (other than group A)	15	9	6	(40)

* Minimum inhibitory concentration.

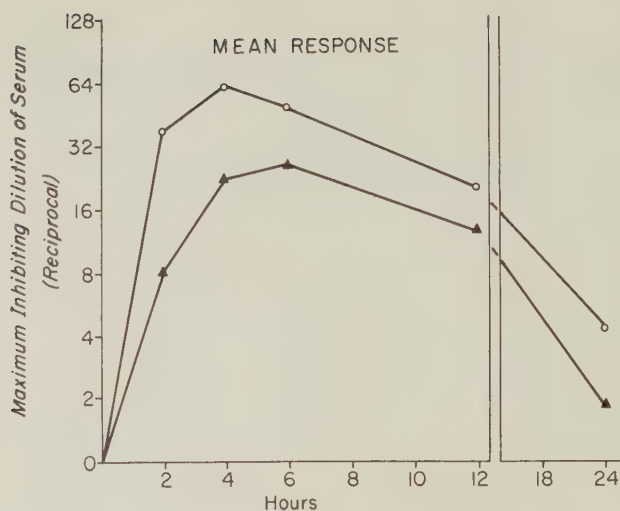


FIG. 1. Average serum inhibiting levels in 10 volunteers after ingestion of 0.5 Gm. tetracycline (O) and 0.3 Gm. demethylchlortetracycline (▲).

80 per cent of the streptococci, and 84 per cent of the coli-aerogenes organisms were more susceptible to demethylchlortetracycline. In no instance was the minimum inhibitory concentration of tetracycline lower than that of demethylchlortetracycline.

Serum Levels. The average serum levels of antistreptococcal activity after the oral administration of the test antibiotics are presented in figure 1. The results are expressed as the maximum number of serum dilutions that inhibited growth. Tetracycline, 0.5 Gm., produced higher levels of activity than 0.3 Gm. of demethylchlortetracycline at all time intervals measured during a 24 hour period.* Complete results of the assays on all 10 subjects after the ingestion of 0.5 Gm. of

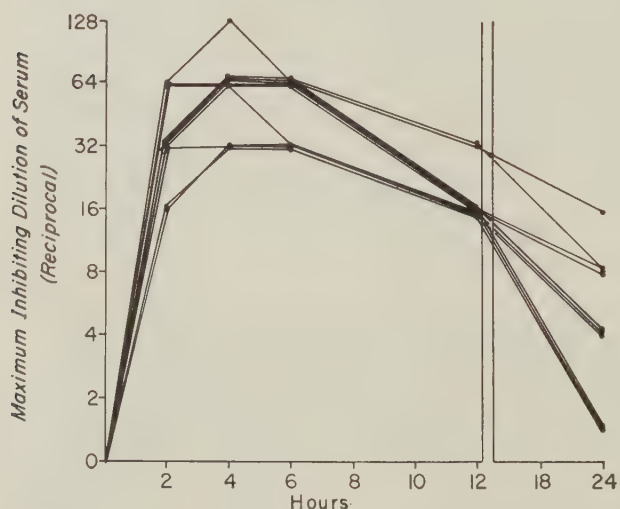
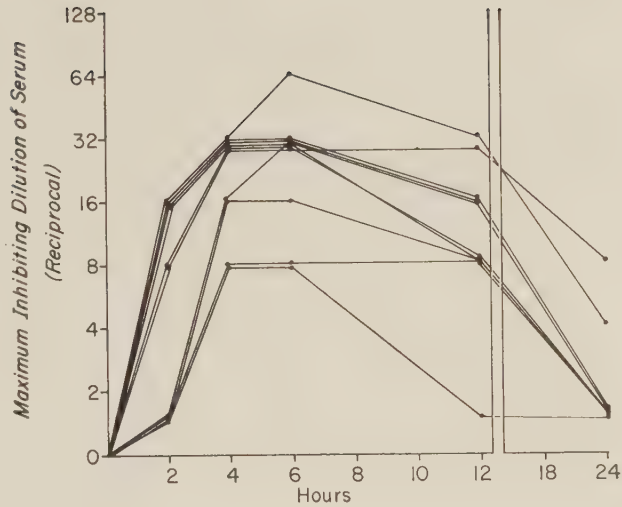


FIG. 2. Inhibiting serum levels in 10 subjects after ingestion of 0.5 Gm. tetracycline. There was prompt absorption with high levels in all instances.

*Assays of the capsules by Lederle Laboratories were as follows: demethylchlortetracycline (150 mg. capsule) assayed at 163.8 mg.; tetracycline with citric acid (250 mg. capsule) 307 mg. and 286 mg.; tetracycline with glucosamine, 301 mg. and 288 mg. Assays of the same capsules performed by Dr. Henry Welch, Food and Drug Administration, gave the following results: Tetracycline with citric acid, 260 mg.; tetracycline with glucosamine, 272 mg.

FIG. 3. Inhibiting serum levels in 10 subjects after ingestion of 0.3 Gm. of demethylchlortetracycline. The levels were somewhat lower, and there was greater variability than with tetracycline.



tetracycline are presented in figure 2. There was a prompt and consistently high level of activity in all of the subjects. Figure 3 shows the results in the same subjects after ingestion of 0.3 Gm. of demethylchlortetracycline. Here the responses showed greater variability and somewhat lower levels.

Each subject was asked to report any ill effects after taking the antibiotics. With 0.5 Gm. of tetracycline only 1 subject experienced nausea, and this persisted for about 30 minutes. With 0.3 Gm. of demethylchlortetracycline this same subject had nausea lasting 12 hours. Three other subjects experienced nausea lasting from 5 to 15 hours, and another 3 had minimal discomfort lasting from a few minutes to one hour. Only 3 subjects had no ill effects after the ingestion of demethylchlortetracycline.

TABLE III
Antibacterial Action of Sera of 13 Patients after 3 to 5 Days of
Demethylchlortetracycline, Using 2 Dosage Schedules

Patient	Dosage	Serum dilution (reciprocal)			
		2	4	6	12
Group I	0.3 Gm. every 12 hours				
P. N.		32	16	8	8
I. S.		32	64	32	8
M. D.		32	32	16	8
Average		32	37	42.7	8
Group II	0.3 Gm. every six hours				
W. L.		64	64	64	64
E. W.		64	64	64	—
E. S.		64	64	64	—
D. E.		128	128	128	—
J. B.		128	128	128	—
J. R.		64	64	—	—
A. L.		128	128	128	—
J. A.		64	64	—	—
L. B.		128	128	128	—
C. H.		128	128	128	—
Average		96	96	(96)	—

TABLE IV

Clinical Response of Patients Treated with Demethylchlortetracycline

Diagnosis	Total no. patients	Results			
		Good	Fair	Poor	Indeterminate
Acute bacterial pneumonia	30	21	3	2	4
Acute bronchitis	6	6	0	0	0
Urinary tract infection	4	1	0	0	3
Other (see text)	7	0	0	0	7

Serum assays carried out in 13 patients are presented in table III. The sera were collected after at least three days of therapy and, in most instances, on the fifth and sixth days. The first 3 patients received 0.6 Gm./day of demethylchlortetracycline, and the remaining 10 received 1.2 Gm./day. In the 3 patients who received 0.3 Gm. every 12 hours, the serum activity at 12 hours was no higher than the activity obtained in volunteers after a single 0.3 Gm. dose. In the patients who received 0.3 Gm. every six hours, the average level at each time interval was three times higher than in those who received the drug twice a day.

Clinical Results. The responses of 47 patients treated with demethylchlortetracycline are presented in table IV. Patients with acute bacterial pneumonia generally responded promptly to therapy, and all but 2 were afebrile in from two to seven days. These 2 patients were both chronic alcoholics, and both became afebrile on the tenth day. Of the 3 responses that were considered to be fair, 1 died on the fourth day from cor pulmonale, and a second was responding slowly when chloramphenicol was added on the fourth day of therapy. The third patient developed nausea and vomiting and demethylchlortetracycline was stopped after three days. Two responses were considered to be poor. One patient failed to improve clinically, and roentgenograms showed progression on 0.6 Gm./day demethylchlortetracycline. The other died after eight days of therapy with what was probably a superinfection. He was a 73 year old man who had cerebral infarction complicating his illness. Of the 4 patients who had indeterminate responses, 2 received more than one antibiotic and 2 were changed to other antibiotics after side effects became troublesome. The patients with acute bronchitis all responded well to therapy. Three received 0.6 Gm./day of demethylchlortetracycline, and 3 received 1.2 Gm./day.

TABLE V

Toxic Effects of Demethylchlortetracycline in 47 Patients

Group	No. of patients	Daily dose, Gm.	No. of patients with side effects*				
			A	B	C	D	Total
I	18	0.6	0	0	0	0	0
II	29	1.2	2	2	2	5	11 (38 per cent)

* A = nausea; B = nausea and vomiting; C = nausea, vomiting, and diarrhea; and D = diarrhea only.

Of the patients with acute urinary tract infections, 1 had a good response. The results in the others were indeterminate because 1 patient received multiple antibiotics, 1 patient did not tolerate demethylchlortetracycline, and a third patient had equivocal improvement.

Seven patients who initially were treated with demethylchlortetracycline had illnesses that proved to be other than acute bacterial infections. Three had tuberculosis, 2 had carcinoma of the lung, 1 a pleural effusion, and 1 gangrene of the leg.

Side Effects. Fifteen of the 47 patients complained of some ill effects, and, in 11, the ill effects were probably related to demethylchlortetracycline therapy. Table V summarizes the gastrointestinal disturbances observed. None of the 18 patients who received 0.6 Gm./day complained of ill effects that could be related to demethylchlortetracycline. However, of the 29 patients who received 1.2 Gm./day, 11 (38 per cent) developed gastrointestinal symptoms. In 5 of these the antibiotic had to be stopped and a different antibiotic instituted within the first few days of therapy. In another 5 the medication was continued and the symptoms subsided rapidly. The remaining patient had persistent symptoms of intermittent vomiting and anorexia, but this patient received both chloramphenicol and demethylchlortetracycline.

DISCUSSION

The results reported by Sweeney et al.,² Kunin and Finland,³ and Hirsch and Finland⁴ suggest that tetracycline is absorbed slightly better than demethylchlortetracycline, but that demethylchlortetracycline in equivalent doses produces higher serum levels of activity, because it is more active against the test organisms and is excreted less rapidly than tetracycline. The present studies confirm that demethylchlortetracycline is more active in vitro against most of the organisms than is tetracycline. However, 0.3 Gm. of demethylchlortetracycline produced less serum antibacterial activity in the volunteers than 0.5 Gm. of tetracycline. It is evident that the greater activity of demethylchlortetracycline against the test organism was not adequate to compensate for the smaller amount ingested.

The results of the clinical trials show that demethylchlortetracycline had effective antibacterial action in vivo. A good response was noted in almost all patients in whom a response to the antibiotic could have been expected. The results are comparable to our own past experiences with other tetracyclines.^{8,9} Early in the study patients were treated with 0.6 Gm. demethylchlortetracycline daily. When it became apparent from the blood level studies that 1.2 Gm. was more likely to be comparable to 2 Gm. of tetracycline, patients with acute bacterial pneumonia were treated with 0.3 Gm. of demethylchlortetracycline every six hours. Side effects were common with this increased dosage. Thirty-eight per cent of 29 patients suffered gastrointestinal symptoms, and, in 5 (17 per cent), it was necessary to discontinue the antibiotic. This relatively high incidence of side effects is in contrast to an earlier study at the same hospital, in which only 10 per cent of patients (6 of 60) receiving 2 Gm. daily of tetracycline noted side effects, and the drug was discontinued in only 1 (1.6 per cent). It would appear that gastrointestinal disturbances might limit the usefulness of demethylchlortetracycline in the therapy of severe infections in which high doses are needed.

SUMMARY

A comparison of the in vitro activities of demethylchlortetracycline and tetracycline showed that demethylchlortetracycline was at least as active against all organisms tested and more active than tetracycline against 69 per cent of the strains. Crossover studies in 10 volunteers showed that a single dose of 0.3 Gm. of demethylchlortetracycline gave serum antibacterial levels that were consistently lower than levels obtained with 0.5 Gm. of tetracycline. The more potent activity of demethylchlortetracycline did not fully compensate for the reduced dosage given. Seven of the 10 subjects noted gastrointestinal side effects with demethylchlortetracycline, as opposed to 1 with tetracycline.

In 40 patients with acute bacterial infections the response to demethylchlortetracycline was satisfactory and comparable to earlier results with other tetracyclines. Side effects were common (38 per cent) when 0.3 Gm. was given every six hours.

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Demethylchlortetracycline in the Treatment of Pneumonia

C. JAMES DUKE, SOL KATZ, AND ROBERT F. DONOHUE

Pulmonary Disease Division and the Georgetown Medical Division, District of Columbia General Hospital, and Veterans Administration Hospital, Washington, D. C.

The tetracycline group of antibiotics has achieved a status of great respect and importance in the therapy of infectious diseases because of their reliable efficacy, excellent patient acceptance, and virtual absence of toxicity. A new tetracycline, demethylchlortetracycline,* has been reported¹⁻³ to produce higher and more prolonged blood levels than equivalent amounts of the standard tetracyclines. It also exhibited greater antibacterial activity in vitro than other tetracyclines against several test organisms. Thus, the laboratory superiority of demethylchlortetracycline over the older tetracyclines has been established in quantitative terms. Clinical studies must now establish the in vivo effectiveness and toxic potential of the new drug.

Bacterial pneumonias provide an excellent background against which to evaluate an antibiotic because of the relative precision and certainty with which the various manifestations may be sequentially observed. This report embodies the experience from the treatment of 32 cases of bacterial pneumonia with demethylchlortetracycline. A previous investigation⁴ by one of the authors provides the experience for an over-all comparison of demethylchlortetracycline and the standard tetracyclines in the treatment of pneumonia.

METHODS

Patients were selected for treatment with demethylchlortetracycline by the admitting ward physician of a general medical service on the basis of clinical and radiographic features compatible with an acute bacterial pneumonia. The ward physicians excluded those patients who required parenteral antibiotic therapy because of severity of the illness; also deleted from the study were patients in whom a purulent pleural complication was detected on admission or whose stained sputum smear revealed organisms not commonly susceptible to tetracycline therapy.

Initial laboratory studies obtained in each case included a hemogram, urinalysis, sputum smear and culture, and, in most instances, blood cultures. Chest roentgenograms were obtained on admission and at appropriate intervals thereafter. Pleural fluid and bronchial secretions were cultured in several cases. Demethylchlortetracycline was administered orally in a dose of 125 mg. every six hours, although several patients received 250 mg. as an initial dose and 1 patient received 250 mg.

This study was performed during the tenure of a Resident Fellowship of the American Trudeau Society (Medical Section of National Tuberculosis Association) (Dr. Duke).

*The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin. The demethylchlortetracycline used in this study was generously supplied by Dr. Stanton Hardy, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I
Detailed Bacteriological Data: Group I

	Sputum	Blood	Sputum and blood
<i>D. pneumoniae</i>	4	3	1
{ <i>D. pneumoniae</i>	1	0	0
{ <i>A. aerogenes</i>			
{ <i>H. influenzae</i>	1		
{Alpha <i>Streptococcus</i>		1	
Mixed flora	4	0	0

every six hours. Antibiotic combinations were not employed in any case, nor had any patients received prior antibiotic therapy for the infection under study.

Evaluation of the therapeutic effect of demethylchlortetracycline was facilitated by grouping patients according to the presence or absence of complications or associated bronchopulmonary disorders. Group I consisted exclusively of uncomplicated pneumonias. Group II included those patients in whom pleural effusion, bronchial obstruction, or suppurative complications were present at the start of treatment or shortly thereafter. Group III was composed of patients in whom structural bronchopulmonary disease antedated the acute infection.

The response to therapy of patients in group I was graded as excellent, good, fair, or unsatisfactory, and was based on rapidity of symptomatic relief, decline of fever and leukocytosis, and rate and degree of radiographic change. Groups II and III were more difficult to assess in regard to therapeutic result. These patients developed local complications of pneumonia (group II) or had pre-existing underlying bronchopulmonary disease (group III). Such associated factors commonly modify the over-all response to treatment. They should be kept in proper perspective and given adequate consideration for accurate appraisal of drug effect. Because the

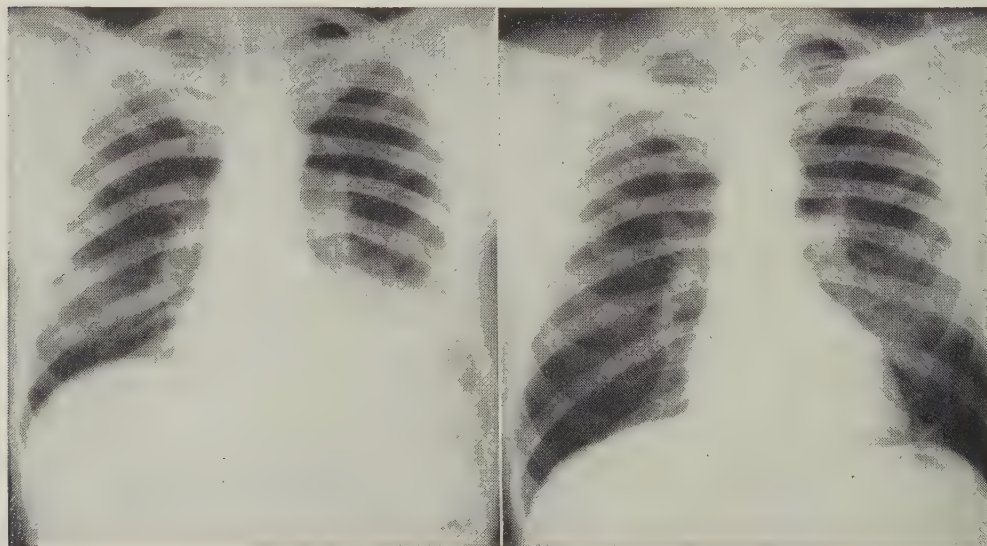


FIG. 1. Uncomplicated left lower lobe pneumococcal pneumonia (left) showing prompt response to demethylchlortetracycline (right).

TABLE II
Detailed Bacteriological Data: Group II

	Sputum	Blood	Sputum and blood
<i>D. pneumoniae</i>	3	0	2
Beta <i>Streptococcus</i> , mixed flora	1	0	0
<i>Staph. aureus</i> , coagu- lase negative, mixed flora	1	0	0
Mixed flora	2	0	0

cases constituting groups II and III were somewhat more complex than those of group I, they were graded as satisfactory or unsatisfactory in regard to therapeutic response.

RESULTS

Fifteen patients were suitable for inclusion in group I, the uncomplicated pneumonias. Twelve were men, and the average age was 36.7 years. Sixty-seven per cent of this group had a specific bacterial pathogen isolated from sputum and/or blood. A mixed flora, consisting of alpha *Streptococcus*, *Neisseria catarrhalis*, *Neisseria sicca*, and *Hemophilus hemolyticus*, was obtained from the remainder of this group. *Diplococcus pneumoniae* was the principal pathogen in 53 per cent of cases and was an associated pathogen in 1 case. Detailed bacteriological data for group I are presented in table I. The pneumonic process was unilobar in 14 instances and multilobar in 1 case (fig. 1).

The response to demethylchlortetracycline therapy for group I was judged to be excellent in 11 cases, good in 2, and fair in 1. The fair response occurred in a patient with pneumonia due to *H. influenza* and alpha *Streptococcus*. One patient in group I was not classified regarding therapeutic response but the case is of sufficient interest to warrant more detailed discussion:

E. M., a 58 year old man, was hospitalized with classical features of pneumococcal pneumonia. *D. pneumoniae* was subsequently isolated from three blood cultures obtained on admission. The initial response to demethylchlortetracycline was prompt and dramatic in terms of subjective improvement and remission of fever. On the fourth hospital day, however, the temperature rose again, and by the sixth day, the patient was seriously ill and toxic. Sputum culture obtained at this time later yielded a tetracycline-resistant *Staphylococcus aureus*; a repeat chest roentgenogram revealed a new area of consolidation in the contralateral lung with associated pleural effusion. In spite of a suitable change in antibiotic therapy, the patient rapidly deteriorated and died on the ninth hospital day.

Group II consisted of 9 patients, all men. Inclusion in this group was based on the presence of complications, as outlined in the preceding section. The average age for these patients was 42.9 years. *D. pneumoniae* was the etiological agent in 55 per cent of the cases in group II; organisms of uncertain significance, namely, a coagulase-negative *Staph. aureus* and a beta *Streptococcus*, were isolated in individual cases in only one of numerous cultural studies. The detailed bacteriological data for group II are presented in table II.

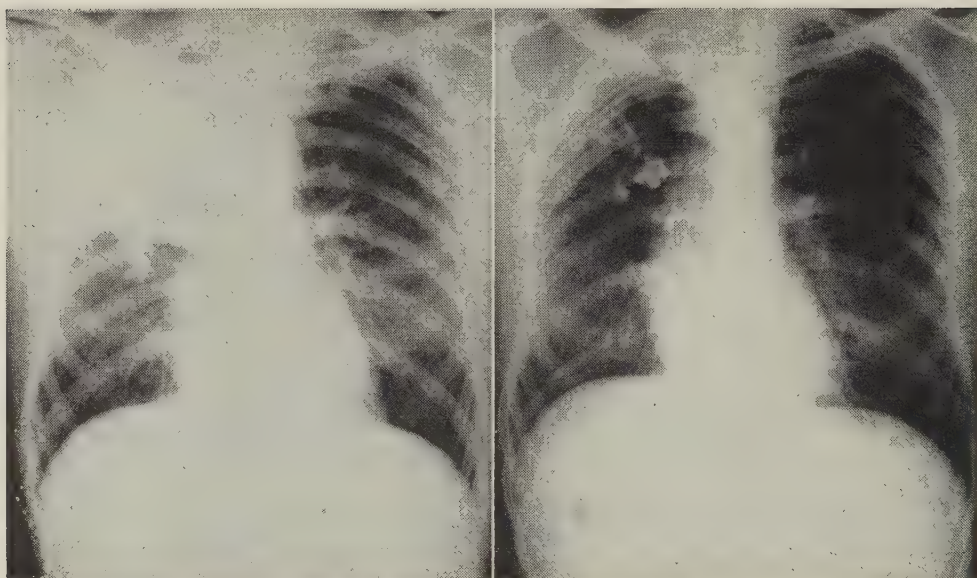


FIG. 2. Right upper lobe lung abscess (left) successfully treated with demethylchlortetracycline (right).

The complications characterizing this group; pleural, bronchial, and suppurative, were present singly and in various combinations in individual cases. One patient had a large lung abscess (fig. 2), and 2 had loculated serous pleural effusions, one of which was associated with lobar atelectasis. Another patient had a moderate degree of parietal pleural effusion plus extensive interlobar effusion. Virtually complete lower lobe atelectasis was noted in 2 patients, while 2 others had delayed pneumonic resolution with radiographic evidence of intrinsic parenchymal shrinkage, fibrosis, and highlight formation, the signs of a destructive or necrotizing pneumonia.

The response of these patients to demethylchlortetracycline therapy was at least satisfactory in terms of subjective relief, decline of fever, and leukocyte response. The rate of radiographic improvement consistently lagged behind these other features because of the mechanical factors involved. Actually, the response, except for roentgenographic tardiness, was usually very good and not adequately portrayed by the term "satisfactory."

One patient in group II had no response, but rather progression of the disease, during 3½ days of demethylchlortetracycline therapy. This case was marked by extensive parenchymal and pleural involvement, but a specific organism was not recovered in spite of intensive bacteriological study. Penicillin was similarly ineffective in this case, with extremely slow improvement being noted after the combination of penicillin and chloramphenicol was employed.

Eight patients were classified in group III because of chronic underlying structural bronchopulmonary disease. Half of this group were men, and the average age was 53 years. The bacteriological studies in this group were in sharp contrast to those for groups I and II, only 1 patient yielding a pathogenic organism culturally. *D. pneumoniae* was cultured from both the blood and sputum of 1 patient,

whereas 6 patients had mixed flora in the sputum. The culture reports on the remaining patient were not available.

A variety of underlying structural bronchopulmonary disorders were encountered in this group of patients. Two patients had a background of extensive fibrotic inactive pulmonary tuberculosis. One of these patients developed a prominent pleural component related to the acute bacterial infection. The 2 oldest patients in the study (aged 85 and 89 years, respectively) had radiographic evidence of diffuse pulmonary fibrosis, and 1 had, in addition, other parenchymal infiltrations suggestive of tuberculosis of uncertain activity. Another patient had extensive fibrocystic parenchymal changes due to sarcoidosis (fig. 3). Two patients had classic findings of chronic obstructive emphysema, and still another patient had had six previous episodes of pneumonia.

The therapeutic response to demethylchlortetracycline in this group of patients was very satisfactory in all cases in spite of the tendency of pneumonia in patients with chronic lung disease to resolve more slowly and to develop complications more frequently than in those with otherwise basically normal lungs. As in group II (pneumonia plus complications), the clinical response of the patients in group III was consistently prompt and very satisfactory in degree, although the radiographic response was slower than is customarily observed in acute pneumonias.

The over-all results in treating 32 patients having acute bacterial pulmonary infections with demethylchlortetracycline show that a satisfactory response was obtained in all except 2 patients. Both of these patients had infection due to organisms not susceptible to tetracycline therapy, in 1 patient a *Staphylococcus* and in the other an unidentified organism that caused a progressive parenchymal and pleural process, which was subsequently unresponsive to penicillin and only slowly responsive to the combination of penicillin and chloramphenicol. In 59 per cent of the patients studied a specific bacteriological agent was isolated, with *D. pneumoniae* accounting for 47 per cent of the total.

Patient acceptance of demethylchlortetracycline was excellent, with no instances

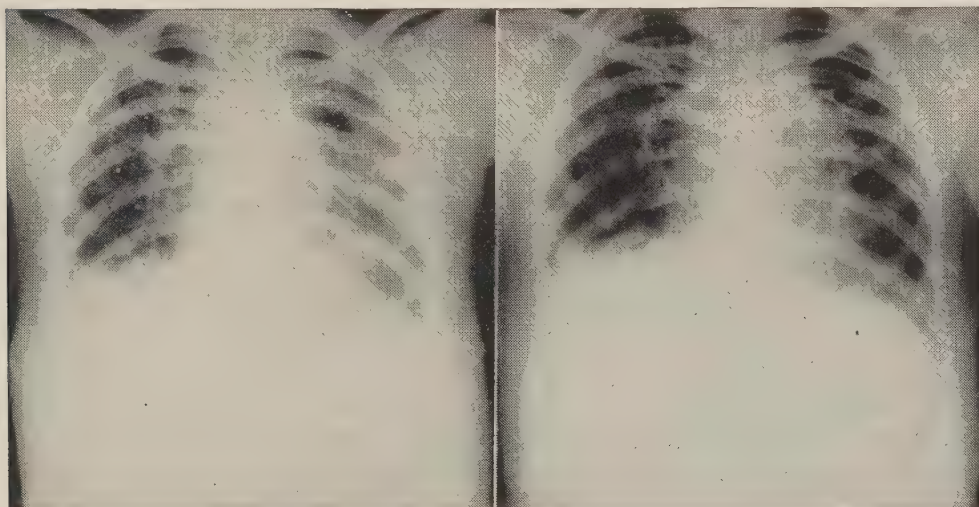


FIG. 3. Bronchopneumonia in a patient with advanced fibrocystic sarcoidosis (left) showing good response to demethylchlortetracycline (right).

of gastric or bowel intolerance. As might be anticipated in tetracycline therapy, sensitivity reactions were not encountered. The occurrence of a staphylococcal pneumonia during the course of apparently successful treatment of a pneumococcal pneumonia may have been an instance of superinfection related to broad-spectrum antibiotic therapy but may just as reasonably be considered as a hazard peculiar to the hospital environment.

DISCUSSION

Demethylchlortetracycline was found to be an effective and safe antibacterial agent on the basis of the observations made during this study. Successful therapy of such a group of acutely ill patients with pneumonia poses a severe challenge to any antibiotic drug, and the particular features of the pneumonias, chiefly the radiographic changes and clinical response, permit a rather precise evaluation of the therapeutic result. The consistency of the satisfactory responses to demethylchlortetracycline, both clinical and radiographic, observed during this study strongly supports those earlier investigations that announced the potential of this new tetracycline.

The system of grouping patients employed in this report, that is, classification according to the presence or absence of complications or underlying chronic lung disease, was of considerable value because it aided in evaluating drug effect while keeping in proper perspective those strictly mechanical factors (complications or antecedent lung disease) that tended to obscure drug effect. Thus, the complications and structural changes encountered in patients of groups II and III are properly considered as mechanical impediments to normal resolution and must, therefore, be taken into account when judging the results of therapy. Actually, vigorous utilization of mechanical therapeutic procedures, such as thoracentesis and bronchoscopy, can be expected to promote more rapid recovery in the complicated cases.

Patient tolerance of demethylchlortetracycline was excellent with the dosage used in this study. This is consistent with the general experience that bothersome gastrointestinal side effects of the tetracycline drugs are closely related to the dosage used. Some observers have pointed out that in order to achieve maximum therapeutic effect with the tetracyclines, prohibitive gastrointestinal disturbances were encountered because of the large dosages required. Demethylchlortetracycline gives promise of overcoming this deficiency in view of the high and prolonged blood levels attainable with relatively small dosages, while simultaneously effecting a highly satisfactory therapeutic result.

The next important consideration regarding demethylchlortetracycline concerns its usefulness and merits as compared to the other tetracyclines and the standard of reference for all antibiotics, penicillin. While this study is not of a comparative nature and thus not a completely suitable vehicle for such comparison, we believe that an extensive past experience in treating the pneumonias provides suitable background for drawing certain conclusions. Although others have been unable to demonstrate a definite superiority of penicillin over tetracycline in the treatment of pneumonia, we believe that penicillin is somewhat more effective. Demethylchlortetracycline seems more nearly to approach the effectiveness of penicillin than the

other tetracyclines. This seeming superiority of penicillin over tetracycline and of demethylchlortetracycline over the older tetracyclines is merely one of degree, a superiority which defies more explicit definition in quantitative terms. Practically, the difference in therapeutic effectiveness of these drugs is not important in pneumococcal pneumonia.

SUMMARY

Thirty-two patients with acute bacterial pulmonary infections were treated with a new tetracycline, demethylchlortetracycline. Satisfactory results were obtained in all except 2 patients.

The comparative efficacy of demethylchlortetracycline, the older tetracyclines, and penicillin is discussed. Demethylchlortetracycline is believed to approach penicillin more nearly than the other tetracyclines in therapeutic activity.

On the basis of this study, demethylchlortetracycline can be recommended in the treatment of acute bacterial pneumonias due to susceptible organisms.

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Demethylchlortetracycline in Genitourinary Infections

MYRON S. ROBERTS, HARRY SENECA, JOHN K. LATTIMER, P. MILNER,
P. PEER, AND O. K. TROC

*Columbia University College of Physicians and Surgeons, The Squier Urological Clinic,
Presbyterian and Babies Hospital, Medical Center, New York, N. Y.*

Demethylchlortetracycline (Declomycin*) was first isolated by McCormick et al¹ in 1957. It was produced by a mutant strain of *Streptomyces aureofaciens*. Since its introduction laboratory investigations have been performed by Sweeney et al² and Kunin and Finland.³ Because of their favorable reports a clinical study of this drug for efficacy against urinary infection was begun at the Squier Urological Clinic. Demethylchlortetracycline has been used in the treatment of 75 patients with genitourinary infections to date.

PHARMACOLOGY

Structurally demethylchlortetracycline is very similar to tetracycline and chlortetracycline, differing from the latter by the absence of a methyl group in position 6 (fig. 1). It is readily absorbed from the intestinal tract with peak serum concentrations in two to four hours.

It produces a much higher antibacterial activity in the serum after oral administration than either chlortetracycline or oxytetracycline. It is slowly excreted by the kidneys with serum antibiotic activity present as long as 120 hours after administration.

In vitro studies show the bacterial spectrum of demethylchlortetracycline to be similar to that of tetracycline, with demethylchlortetracycline being more effective. Strains resistant to tetracycline were also resistant to demethylchlortetracycline. The results of the minimal inhibitory concentration studies of demethylchlortetracycline and tetracycline, using stock pathogens, are shown in table I. Demethylchlortetracycline is about two tubes more effective than tetracycline.

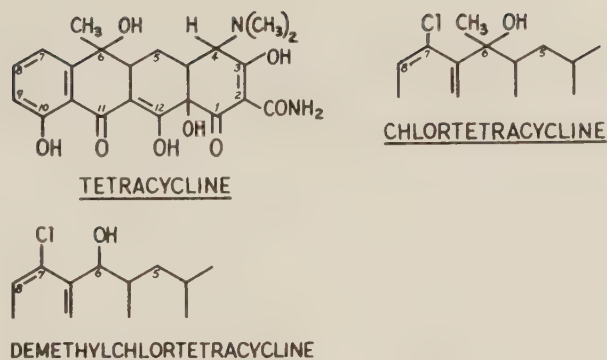
METHOD

Seventy-five patients with genitourinary infections were treated with demethylchlortetracycline. Both hospitalized and outpatient subjects were treated. A carefully catheterized urine culture with drug susceptibility study, using the tube dilution method, was obtained before starting therapy. The patient was then instructed to take 900 mg. of demethylchlortetracycline per day, in three divided doses, after meals, for six days. On the seventh day, the patient was carefully questioned as to subjective clinical improvement and signs of toxicity. A repeat urine culture, with drug susceptibility studies, was obtained at that time.

Supported by a grant from Lederle Laboratories Division, American Cyanamid Co.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin.

FIG. 1. Structural formulae of tetracycline, chlortetracycline, and demethylchlortetracycline.



RESULTS

Seventy-five pretreatment urine cultures were obtained. Of these 61 were positive and 14 revealed no growth despite the presence of urinary symptoms. One patient stopped the medication after two days because of severe nausea and vomiting. Six cultures grew out two organisms each, so that 66 organisms were cultured initially from these 75 patients for evaluation. Two patients whose initial cultures showed no growth developed positive cultures while on therapy.

Table II shows the bacteria cultured and those cultures that became sterile after six days of demethylchlortetracycline therapy. It was interesting to note the frequency of the appearance of *Aerobacter aerogenes* and its relatively greater resistance to antibiotics. *Staphylococcus* and enterococcus were very susceptible to demethylchlortetracycline. Thirty-three per cent of all cultures became sterile after therapy, regardless of organism.

The 66 positive cultures were then divided into gram-positive and gram-negative groupings to determine the effects of demethylchlortetracycline in each. It was effective in producing a sterile culture in 91 per cent of the infecting gram-positive organisms and in 12 per cent of the gram-negative organisms (see table III).

Next we evaluated the effect of demethylchlortetracycline in the treatment of acute and chronic urinary tract infections. As might be anticipated, the acute infections responded more favorably. There were four positive urine cultures from

TABLE I

Antibacterial Spectrum of Demethylchlortetracycline and Tetracycline, Expressed in $\mu\text{g./ml.}$

	Demethylchlortetracycline	Tetracycline
<i>Bacillus proteus</i> (Reilly)	50	>100
<i>Pseudomonas aeruginosa</i> (Jones)	1.56	25.0
<i>Aerobacter aerogenes</i> (Pinkerton)	50.00	>100.00
<i>Salmonella paratyphi</i> (ETS Army)	0.19	0.78
<i>Klebsiella pneumoniae</i> (type I Army)	<0.19	<0.19
<i>Escherichia coli</i> (0126 Army)	0.39	0.78
Enterococcus (Lubin)	6.25	1.56
<i>Micrococcus pyogenes</i> var. <i>aureus</i> Y-1	<0.19	<0.19
<i>Streptococcus pyogenes</i> C203MV	<0.19	<0.19

TABLE II

Results with 66 Pathogens Cultured from Patients Treated for 6 Days with Demethylchlortetracycline

Organism	Number cultured		Number sterile after therapy	
	Number	Per cent	Number	Per cent
<i>Aerogenes</i>	32	48	5	16
<i>E. coli</i>	10	15	5	50
<i>Proteus</i>	8	12	1	12.5
<i>Staphylococcus</i>	7	10.5	7	100
<i>Pseudomonas</i>	3	5	1	33
<i>Enterococcus</i>	3	5	3	100
<i>Escherichia intermedia</i>	1	1.5	0	0
Hemolytic <i>Alkaligenes</i>	1	1.5	0	0
Unidentified gram-positive rods	1	1.5	0	0

A total of 33.3 per cent of all cultures became sterile after demethylchlortetracycline therapy.

TABLE III

Effect of Demethylchlortetracycline on Gram-positive and Gram-negative Pathogens

Culture	Number cultured		Number sterile after therapy	
	Number	Per cent	Number	Per cent
Gram positive	11	17	10	91
Gram negative	55	83	12	22
Gram negative excluding <i>E. coli</i>	45	80	7	13

TABLE IV

Results of Acute and Chronic Infections Treated with Demethylchlortetracycline

Culture	Number cultured		Number sterile after therapy	
	Number	Per cent	Number	Per cent
Acute	4	6	4	100
Chronic	62	94	18	27

TABLE V

*The in Vitro Susceptibility of Pathogens from 60 Patients to Demethylchlortetracycline and Tetracycline**

	No. of patients	Per cent
Demethylchlortetracycline and tetracycline equal	24	40
Demethylchlortetracycline superior to tetracycline	36	60
1 tube better	27	45
2 tubes better	7	12
3 tubes better	2	3

* Not one drug susceptibility study showed tetracycline to be superior to demethylchlortetracycline.

TABLE VI

Side Effects of Demethylchlortetracycline Among 75 Patients

Side effect	No. of patients
Gastrointestinal	
Nausea	6
Nausea and vomiting	9
Dyspepsia	1
Total	16
Skin reactions	
Dermatitis medicamentosa	1
Pruritis vulvae	1
Total	2
Total	18 (24%)

patients with acute infections. All four became sterile after therapy. Of the remaining 62 cultures from chronic urinary tract infections, 18 (27 per cent) responded to treatment (see table IV).

Drug susceptibility studies, using the serial tube dilution method, were performed on 60 of the 66 positive cultures. Comparing the activity of demethylchlortetracycline to that of tetracycline, we found the susceptibility greater to demethylchlortetracycline in 60 per cent of the studies, and equal in 40 per cent (see table V). No drug susceptibility study was found where an organism was more susceptible to tetracycline than to demethylchlortetracycline.

Of interest were the post-treatment drug susceptibility studies. If the infected urine was not sterilized by the demethylchlortetracycline, all susceptibility to this drug was lost when post-treatment studies were done. There was an apparent cross resistance, which developed not only to demethylchlortetracycline, but to tetracycline as well. This was true in almost every instance.

Thirteen patients had 55 determinations of serum antibiotic activity levels during therapy. The specimens were assayed by the *Bacillus cereus* agar diffusion method and compared with responses obtained with serum prepared to contain known amounts of demethylchlortetracycline and tetracycline. The tetracycline serum antibiotic activity was expressed in tetracycline equivalents, which means the tetracycline concentration required for equivalent demethylchlortetracycline activity in µg./ml. The results showed that the tetracycline equivalents were about three times those of demethylchlortetracycline, or that demethylchlortetracycline had three times greater serum antibiotic activity than did tetracycline.

Untoward symptoms occurred in 24 per cent of our patients. One patient (mentioned previously) stopped the medication after two days because of severe nausea and vomiting. Another patient independently decreased the dosage to 150 mg. twice a day because of severe nausea.

Gastrointestinal symptoms accounted for 88 per cent of the toxic reactions. No severe reactions were reported by any of the patients on therapy. Table VI outlines the toxic reactions encountered.

SUMMARY

Demethylchlortetracycline was used in the treatment of 75 patients with symptoms or signs of genitourinary infection. It was effective in rendering the urine sterile in 33 per cent of all cases. It was most effective against infections with the gram-positive cocci, sterilizing the urine in 91 per cent of the patients. It was much less effective against the infections with gram-negative organisms. Acute urinary tract infections responded more favorably than did chronic infections. The serum antibiotic activity level was three times that obtained with tetracycline, and serial tube dilution drug susceptibility studies showed demethylchlortetracycline to be more effective than tetracycline in 60 per cent of the cases. There was not 1 case in which the drug susceptibility was greater to tetracycline than to demethylchlortetracycline. Toxic symptoms, mostly gastrointestinal, were noted in 24 per cent of our patients. No serious side effects were observed. Only 1 patient of the 75 stopped treatment because of toxicity (nausea).

It appears that demethylchlortetracycline is a more effective antibiotic than is tetracycline and will probably play an important role in the treatment of genitourinary infections.

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Therapeutic Evaluation of Demethylchlortetracycline in Human Brucellosis

GUILLERMO CHAVEZ MAX

Instituto Mexicano del Seguro Social, Mexico, D. F.

Since the determination of the structural formula of chlortetracycline, which was isolated by Duggar et al from *Streptomyces aureofaciens*, new antibiotics of the same family have been identified and synthesized.

The latest, and possibly the most important, is demethylchlortetracycline, obtained and identified by McCormick and collaborators¹ in 1957 from a mutant of *S. aureofaciens*.

Chemical and experimental studies carried out by Redin and McCoy² and Sweeney and co-workers³ attributed to this new drug a remarkable stability in acid as well as in alkaline media. The drug differs chemically from similar antibiotics due to the absence of one methyl group in position 6 from the basic tetracycline molecule. The studies of Kunin and Finland⁴ demonstrated its antimicrobial activity, which is similar to that of other tetracyclines. Its low toxicity, its rapid and almost total absorption in the small intestine, and its slow urinary excretion suggest that this drug promises to be of great value in the treatment of bacterial diseases.

In the present work the action of this new antibiotic on human brucellosis is studied, as well as its toxic action on the liver, kidneys, and blood. Undesirable side effects are also evaluated.

MATERIALS AND METHODS

Nine patients were selected who had a clinical, serological, and bacteriological diagnosis of brucellosis (*Brucella melitensis*). There were 1 woman and 8 men patients, ranging in age from 13 to 52 years, and in body weight from 32 to 70 Kg. All patients were subjected to the same experimental conditions in so far as rest and feeding were concerned. The duration of the disease varied in each case: in the first and second cases, 20 days; in the third, 60; in the fourth, 30; in the fifth, 45; in the sixth, 60; in the seventh, 40; in the eighth, 30; and in the ninth, 45 days. Most of the patients had been given low dosages of chloramphenicol for a short time, and thus the symptomatology was modified in some cases or temporarily absent in others. Six patients (cases 2, 4, 5, 6, 7, and 9) were treated when the condition was re-established.

In most cases, treatment was established in the subacute phase and in 3 cases, in the acute phase of the disease. Clinical studies were made in all cases at the beginning and at the end of the treatment in order to evaluate the antibiotic action on the liver, kidneys, and blood; for that purpose, blood bilirubin was determined, the cephalin cholesterol test was carried out, as well as the thymol turbidity test, and total proteins were determined. Urea, creatinine, and blood glucose were determined, urine was studied, erythrocytes and hemoglobin in the blood were measured.

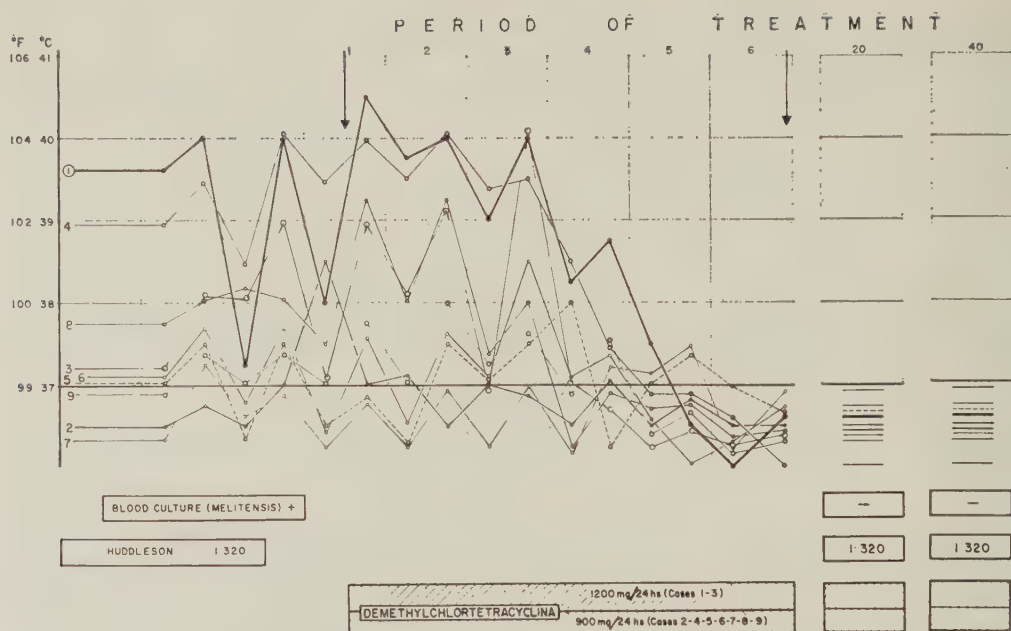


FIG. 1. Temperature curves for patients treated with demethylchlortetracycline.

Agglutination to *Brucella* reaction was investigated and blood cultures were made at the beginning, in the middle, and at the end of treatment.

Based on the experimental chemical studies of previously mentioned investigators, the drug was administered at a dosage of 1200 mg. in 24 hours in the acute cases and in divided doses of 900 mg. in the subacute cases, also in 24 hours. According to Sweeney et al,³ the antibiotic reaches useful antimicrobial levels in the blood.

Since it has been shown that the other tetracyclines are effective only if treatment lasts 40 days, demethylchlortetracycline was administered to our patients for the same period.

RESULTS

In every case, both in the acute phase as well as in the subacute phase, the antibiotic had a suppressive effect on the symptomatology, which was more accentuated and rapid on the temperature curve.

Figure 1 shows the temperature curves for each patient, and it can be seen that they show an increase in the first few hours after the beginning of the treatment and descend progressively and rapidly until the fourth to the fifth day, when they become normal and remain so until the end of treatment.

Demethylchlortetracycline dosage used in the acute cases (1 and 3) was 1200 mg. in 24 hours. In the rest of the patients only 900 mg., in 24 hours, was necessary.

The blood culture was positive for *Br. melitensis* in all cases, when treatment began, but it was negative in further investigations 20 days later and at the end of treatment.

Huddleson's test was positive in all cases at the same dilutions from the beginning to the end of treatment. Diaphoresis, asthenia, adynamia, and anorexia disap-

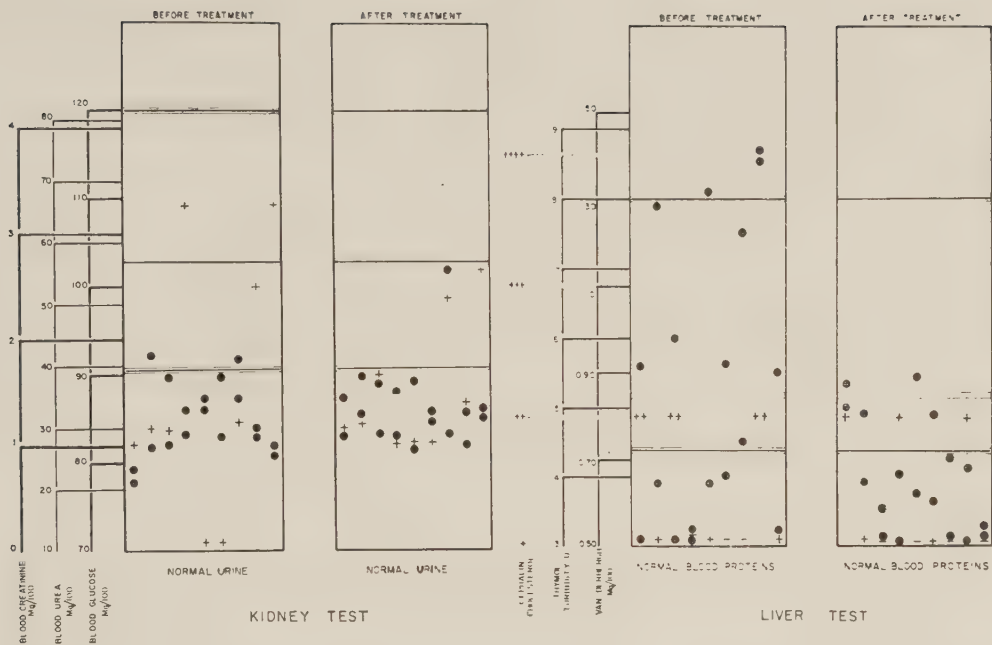


FIG. 2. Results of kidney and liver tests in patients treated with demethylchlortetracycline.

peared at the latest on the tenth day, and only in patients 1, 7, and 8 was there occasional nausea and vomiting between the twentieth and twenty-fifth days, which stopped after the antibiotic was administered in milk. In every case, splenomegaly was clinically and radiologically proved, and it disappeared after the fifteenth day of treatment.

Patient 1 had articular pains with phlogosis when the treatment was established, but they disappeared by the twentieth day. Patient 5 had pain in the area of the sciatic nerve up to the twenty-fifth day of administration of the drug.

Clinical observation of the patients under study showed no alterations of hepatic and renal function. The laboratory studies used to estimate the activity of the liver and kidneys were those usually employed in general clinical investigations. The functioning of these organs was normal or was within the higher limit of normal.

Figure 2 shows the statistical data obtained from renal and hepatic function tests at the beginning and at the end of the treatment. In the chart of renal tests, normal urea, creatinine, and glucose, as well as normal urine, are shown. The hepatic tests were also normal.

CONCLUSIONS

1. Demethylchlortetracycline in dosages of 900 to 1200 mg./24 hours continued for 40 days showed a suppressive action on the symptoms of brucellosis.
2. The temperature increases a few hours after first administration of the antibiotic and becomes normal four or five days later.
3. None of the undesirable reactions of the drug necessitated interruption of the treatment. The gastric irritation symptoms were eliminated by administering the drug in milk.

4. The blood cultures, which were positive for *Brucella* before treatment was begun, became negative in subsequent investigations.

5. The agglutination indexes show no modification during treatment.

6. Up to the time of this report none of the treated patients has shown signs of relapse, either clinically or bacteriologically.

7. Demethylchlortetracycline was effective without addition of any other drug.

The time that has elapsed since the termination of treatment in the first case is two and a half months; in the most recent case, one month. This period is too short to permit definitive conclusions regarding the superiority of demethylchlortetracycline over other tetracyclines having a similar action on human brucellosis. The clinical and bacteriological follow-up studies that we make in apparently cured cases will allow us in the future to report more conclusive data. In any event, we may state at this time that demethylchlortetracycline has the advantage over other drugs of the same group in being useful at lower dosage and in being better tolerated.

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Clinical Results with Demethylchlortetracycline in Pediatrics in Japan and Comparative Studies with Other Tetracyclines

RYOCHI FUJII, HARUO ICHIHASHI, MIKIO MINAMITANI,
MASATOSHI KONNO, AND TOMOKO ISHIBASHI

Department of Pediatrics, Tokyo University Branch Hospital, Tokyo, Japan

7-Chloro-6-demethylchlortetracycline, which was described by McCormick et al¹ in 1957, has the following advantages over the conventional tetracyclines: it is more stable to acids and alkalis, it is better absorbed from the intestinal canal, there is less excretion from the kidney, and there are consequent higher blood levels and longer retention in the blood.^{2,3}

The authors investigated demethylchlortetracycline in regard to blood level, body accumulation, urinary excretion, and migration into the spinal fluid, as well as therapeutic effectiveness in pediatric infections. These results were compared with those of tetracycline hydrochloride, tetracycline sodium metaphosphate mixture, tetracycline sodium citrate mixture, and chlortetracycline.

MATERIALS AND METHODS

Demethylchlortetracycline blood levels and urinary excretions were measured in 3 healthy adults and 10 healthy children, and the migration into the spinal fluid was determined in 1 pediatric case each of serous meningitis and microcephalia. Clinical effectiveness was studied in 309 cases of acute pediatric infections (75 patients were treated with demethylchlortetracycline; 73, with tetracycline sodium citrate mixture; and 161, with tetracycline sodium metaphosphate mixture).

The concentration of the antibiotics was measured by means of the laying method (primary dimension diffusion) described by Torii and Kawakami.^{4,5} That is, the agar medium containing the test organism and indicator (0.004 per cent methylene blue) was poured into a small test tube (4.0 × 80.0 mm.) and allowed to solidify, after which the test fluid was layered thereon and cultured. Since the section where there was growth of the organism showed change in color of the indicator, the length of the bacterial growth inhibition from the surface of the medium was measured, thus ascertaining the concentration of the antibiotic in the test fluid.

The test organisms used in the present experiment were *Staphylococcus aureus* 209 P and *Escherichia coli* (NIHJ).

Administration of the antibiotics was as follows: To 3 healthy adults demethylchlortetracycline was given with about 50 ml. of water on an empty stomach in a dosage of 300 mg. (2 capsules of 150 mg. each) and again in a dosage of 500 mg. (2 capsules of 250 mg. each) of other tetracyclines. In children demethylchlortetracycline was taken out of the capsules and given at dosages of 10, 15, or 25 mg./Kg. of body weight, and in other children the drug was given at a dosage of 10 mg./Kg. together with a small amount of sugar water.

Therapeutic effectiveness for acute infections in children was studied in 309 outpatients and inpatients at the Department of Pediatrics, Tokyo University Branch

Hospital, during the period from March, 1957, to September, 1959. The dosage was 10 to 20 mg. of demethylchlortetracycline per/Kg. daily, divided in two, at 12 hour intervals or about 30 mg. of the other tetracyclines per Kg. per day, divided into four portions, namely, before each meal and before retiring.^{6, 7} We seldom adopt the six hour regimen, which disturbs children's sleep and has no greater effect on blood level than our method.

The criteria for evaluation of therapeutic effectiveness were as follows: the result was considered remarkably effective when almost all major signs and symptoms were cleared within 48 hours; effective when the main signs and symptoms were either considerably diminished within 48 hours or had completely disappeared within 96 hours; and ineffective in all other cases. Remarkably effective and effective cases are collectively termed "effective" in all the following passages. The terms "effective" and "ineffective" are applied to the controls according to the therapeutic result without administration of antibiotics.

RESULTS

Blood Demethylchlortetracycline Levels. Blood demethylchlortetracycline levels are in terms of concentration of chlortetracycline equivalent in the blood, and blood levels of other tetracyclines are in terms of tetracycline hydrochloride equivalents. The concentration of demethylchlortetracycline in the blood was determined by crossover tests in 3 children (a 4 year, 3 month old boy, 14.9 Kg.; a 5 year, 8 month old girl, 16.8 Kg.; an 8 year, 2 month old boy, 20.8 Kg.), who were given 10 mg., 15 mg., and 25 mg./Kg., the dosage being changed at weekly intervals. The results were as follows: In the first case, the blood levels before and after each 3, 6, 12, and 24 hour period of single oral medication of 10 mg./Kg. were 0, 3.9,

TABLE I
Blood Levels after a Single Dose of Demethylchlortetracycline, Chlortetracycline, and Tetracycline Sodium Metaphosphate Mixture, 15 mg./Kg. (Crossover Study in 3 Children)

	Hours				
	0	3	6	12	24
Demethylchlortetracycline					
T.I., 7 yr., M	0	2.80	1.90	1.62	1.05
M.Y., 7 yr., M	0	1.88	1.70	1.50	0.50
H.Y., 8 yr., F	0	2.08	2.00	1.00	N.D.*
Average	0	2.25	1.87	1.37	0.52
Chlortetracycline					
T.I.	0	3.20	1.50	0.70	N.D.
M.Y.	0	1.85	0.80	N.D.	N.D.
H.Y.	0	2.08	1.10	N.D.	N.D.
Average	0	2.35	1.13	0.23	0
Tetracycline sodium metaphosphate					
T.I.	0	3.00	1.50	N.D.	N.D.
M.Y.	0	1.55	1.00	N.D.	N.D.
H.Y.	0	2.00	0.58	N.D.	N.D.
Average	0	2.18	1.03	0	0

*N.D. = Zone of growth inhibition negative. Test organism—*Escherichia coli* (NIHJ).

TABLE II

Blood Levels after 300 mg. Demethylchlortetracycline and 500 mg. Tetracycline Sodium Metaphosphate Mixture and Sodium Citrate Mixture Orally (Crossover Study in 3 Adults)

Patient	Age, yr.	Sex	Hours					
			0	1	2	4	6	8
Demethylchlortetracycline								
H.I.	35	M	0	1.00	2.78	3.15	3.25	2.85
M.K.	31	M	0	0.80	2.50	2.60	2.50	2.10
M.M.	33	M	0	—	1.50	1.20	1.39	1.05
Average			0	0.90	2.26	2.65	2.38	2.00
Tetracycline sodium metaphosphate								
H.I.			0	2.00	4.10	3.80	3.00	2.00
M.K.			0	1.10	2.90	2.50	2.20	1.80
M.M.			0	—	1.70	1.05	0.80	0.70
Average			0	1.55	2.90	2.45	2.00	1.50
Tetracycline sodium citrate								
H.I.			0	1.90	3.90	3.50	2.10	1.75
M.K.			0	1.50	3.00	2.50	2.40	1.80
M.M.			0	—	1.35	1.80	1.20	0.50
Average			0	1.70	2.75	2.60	1.90	1.35
Tetracycline hydrochloride								
H.I.			0	—	1.80	1.04	0.90	0.62
M.K.			0	—	1.50	1.20	0.92	0.48
M.M.			0	—	0.60	1.00	0.58	N.D.*
Average			0	—	1.30	1.08	0.80	0.55

*N.D. = Zone of growth inhibition negative. Test organism—*E. coli* (NIHJ).

1.5, 1.33, and 0.5 $\mu\text{g./ml.}$ respectively; of 15 mg./Kg., 0, 5.8, 6.8, 2.3, and 1.5; and of 25 mg./Kg., 0, 6.9, 7.0, 5.2, and 3.4 $\mu\text{g./ml.}$; in the second case, with 10 mg./Kg., 0, 1.71, 1.58, 1.4, and 0; with 15 mg./Kg., 0, 2.9, 3.2, 2.8, and 1.2; and with 25 mg./Kg., 0, 3.9, 4.2, 3.8, and 3.8 $\mu\text{g./ml.}$ respectively; in the third case, with 10 mg./Kg., 0, 3.0, 2.5, 1.9, and 0; with 15 mg./Kg., 0, 3.5, 2.8, 2.0, and 1.5; and with 25 mg./Kg., 0, 4.5, 4.2, 4.9, and 3.0 $\mu\text{g./ml.}$ respectively. The average blood demethylchlortetracycline level after the administration of 10 mg./Kg. was 2.87 $\mu\text{g./ml.}$ at three hours with a gradual decline subsequently, until 24 hours when the drug could not be demonstrated in the blood in 2 of 3 cases ($\geq 0.1 \mu\text{g./ml.}$). The blood level with 15 mg./Kg. was 4 $\mu\text{g./ml.}$ or more for three to six hours, and was 1.4 $\mu\text{g./ml.}$ even after 24 hours. During the third to the twelfth hours after 25 mg./Kg. was given, the blood levels were 4 $\mu\text{g./ml.}$ or more, and even at 24 hours were 3.4 $\mu\text{g./ml.}$

The blood levels (crossover test) after a single dose of 15 mg./Kg. of demethylchlortetracycline, chlortetracycline, and tetracycline sodium metaphosphate mixture given to 3 healthy children, 7 to 8 years of age, are presented in table I. There was scarcely any difference noticeable in the peak after three hours. However, demethylchlortetracycline evidently remained in the blood longer than the other two antibiotics.

Blood concentrations were then compared after a single dose of 300 mg. demethylchlortetracycline and a single dose of 500 mg. tetracycline hydrochloride, sodium metaphosphate, and sodium citrate in 3 healthy adults. The results are shown in table II. There were almost no noticeable differences in the peak blood levels of the sodium metaphosphate, sodium citrate, and demethylchlortetracycline,

TABLE III

*Blood Accumulation of Demethylchlortetracycline Given Every 12 Hours
(7.5 mg./Kg. for 4 Children Orally)*

Patient	Age	Hours, $\mu\text{g./ml.}$												
		0	6	12	18	24	30	36	42	48	54	60	66	72
K.W., M	7 yr., 5 mo.	0	1.5	1.0	2.0	1.3	2.2	1.3	2.5	2.0	4.0	2.6	3.8	2.8
I.T., M	8 yr., 3 mo.	0	2.0	1.5	1.8	1.8	1.9	1.5	2.2	2.0	3.5	2.8	3.6	3.0
I.K., F	11 yr.	0	2.0	1.2	2.2	1.7	3.2	2.0	2.0	1.9	3.2	2.5	3.4	2.8
S.I., M	10 yr.	0	1.2	0.5	1.2	1.3	1.5	1.5	1.9	1.5	2.0	1.3	2.8	1.0
Average		0	1.66	1.05	1.8	1.53	2.2	1.58	2.15	1.85	3.18	2.3	3.4	2.4

Test organism—*E. coli* (NIHJ).

whereas their peak blood levels were more than two times higher than that of tetracycline hydrochloride. The peak appeared between the second and fourth hours with the sodium metaphosphate and sodium citrate, while the appearance of the peak with demethylchlortetracycline was somewhat later, namely, between the fourth and sixth hours. The blood level after 500 mg. of the sodium metaphosphate and sodium citrate are equivalent to the blood level after 300 mg. of demethylchlortetracycline.

Body accumulation of demethylchlortetracycline was studied by giving 15 mg./Kg. to 4 children for one day at 12 hour intervals for three successive days. Blood specimens were collected every six hours, just prior to oral administration of the medication. The results are given in table III. Oral administration at 12 hour intervals resulted in a significant degree of body accumulation.

Urinary Excretion. The urinary volume and the concentration of demethylchlortetracycline in the urine were quantitatively determined, and the rate of cumulative urinary recoveries of the antibiotic administered was measured at 2, 6, 12, and 24 hours after oral administration of 15 mg./Kg. of the drug in 3 children (a 7 year, 5 month old boy, an 8 year, 3 month old boy, and a 12 year old boy). Urinary excretions were compared between demethylchlortetracycline and tetracycline so-

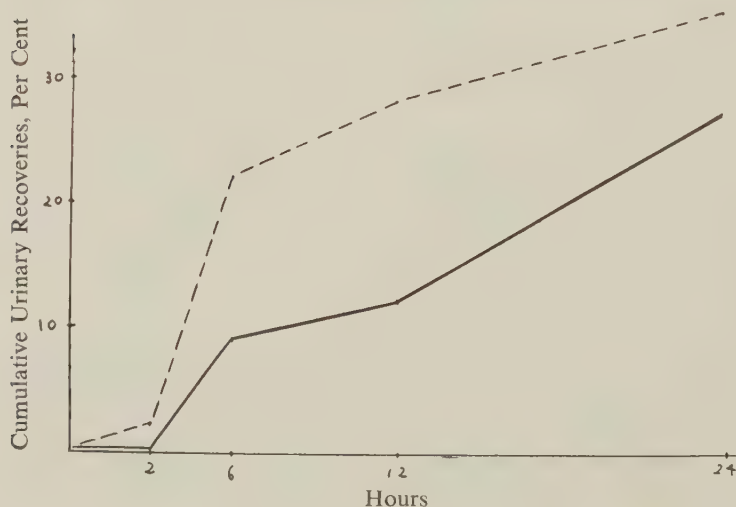


FIG. 1. Urinary excretions of demethylchlortetracycline (—) and tetracycline sodium metaphosphate mixture (---), 15 mg./Kg. Time factors for cumulative recoveries of antibiotics.

dium metaphosphate mixture in crossover study. The results of these determinations are given in figure 1. As can be seen, demethylchlortetracycline showed considerably more inhibition of urinary excretion until 12 hours after administration than the other drug.

Migration into the Spinal Fluid. Concentrations of demethylchlortetracycline in body fluids were determined in 2 children, who were given 25 mg./Kg. of the drug. Blood and spinal fluid samples were collected three hours later. While the blood samples demonstrated the presence of 3.5 µg./ml. and 4.2 µg./ml., respectively, the spinal fluid failed to demonstrate any presence of demethylchlortetracycline (0.1 µg./ml. \geq).

Clinical Results. Therapeutic results of demethylchlortetracycline in 309 pediatric cases of acute infections, including 6 cases of scarlet fever, 137 cases of acute pharyngotonsillitis, 52 cases of acute bronchitis, 12 cases of acute pneumonia, 21 cases of primary atypical pneumonia, 14 cases of bacillary dysentery, 17 cases of infantile diarrhea, 17 cases of acute colitis, 10 cases of furunculosis, and 14 cases of acute lymphadenitis, are summarized in table IV.

Demethylchlortetracycline was effective in 57 (76.0 per cent) of 75 cases; tetracycline sodium citrate mixture was effective in 54 (74.0 per cent) of 73 cases; and tetracycline sodium metaphosphate mixture was effective in 122 (75.8 per cent) of 161 cases. Thus no significant difference was noted among the three drugs. In other words, demethylchlortetracycline, 10 to 20 mg./Kg. (averaging 15 mg./Kg.) daily, in two divided doses, has thus proved almost as therapeutically effective as the other tetracyclines given at 30 mg./Kg. daily in four divided doses. Demethylchlortetracycline was effective in every case of primary atypical pneumonia and bacillary dysentery, which are indications for tetracycline. The type of bacillary dysentery was group B in 10 cases, group D in 4 cases.

TABLE IV

Therapeutic Effectiveness of Demethylchlortetracycline, Tetracycline Sodium Citrate Mixture, and Sodium Metaphosphate Mixture

Diagnosis	No. of cases	Demethylchlortetracycline*			Sodium citrate			Sodium metaphosphate		
		E	I	S	E	I	S	E	I	S
Scarlet fever	6	3	0	0	1	0	0	2	0	0
Acute pharyngitis, tonsillitis	137	24	10	2	27	11	1	47	18	4
Acute bronchitis	52	9	4	0	9	3	0	17	10	1
Acute pneumonia	12	0	0	0	0	0	0	12	0	0
Primary atypical pneumonia	21	2	0	0	3	1	0	11	4	0
Bacillary dysentery	14	5	0	0	1	0	0	8	0	0
Infantile diarrhea	17	2	1	0	4	0	0	6	4	0
Acute colitis	17	2	1	0	4	1	0	8	1	0
Furunculosis	10	3	0	0	0	2	0	3	2	0
Acute lymphadenitis	14	5	2	1	4	1	0	2	0	0
Prophylaxis of secondary infection	9	2	0	0	1	0	0	6	0	0
Total	309	57	18	3	54	19	1	122	39	5
		Effective rate 76.0%		4.0%	Effective rate 74.0%		1.4%	Effective rate 75.8%		3.1%

* Demethylchlortetracycline—10 to 20 mg./Kg./day. Tetracycline sodium citrate and sodium metaphosphate mixtures—30 mg./Kg./day. E = Effective. I = Ineffective. S = Side effects.

TABLE V

Effectiveness of Tetracyclines in Upper Respiratory Tract Infections Due to Viral Etiologies

Secondary infection	Cold agglutination positive			Psittacosis			Hemagglutinating virus of Japan			Influenza A			Adenovirus			Undifferentiated		
	+	-	/	+	-	/	+	-	/	+	-	/	+	-	/	+	-	/
Tetracycline groups																		
Effective	16	17	1	0	5	0	3	5	1	4	0	0	6	5	0	44	0	29
Ineffective	5	2	0	0	1	0	0	0	1	1	1	0	0	3	0	9	0	9
Per cent		82.5			83.3			90.0		66.6			78.5			80.2		
Controls																		
Effective*	6	10	1	0	1	0	0	3	0	4	3	0	0	1	0	33	1	51
Ineffective†	19	33	12	0	2	0	0	6	0	10	9	1	4	1	0	88	4	3
Per cent		33.3			33.3			33.3		25.9			16.7			47.2		

* Identical course shown with tetracycline-effective cases.

† Identical course shown with tetracycline-ineffective cases.

Results of treatment with tetracyclines in 86 cases of acute upper respiratory tract infections where isolation of the pathogens was possible are as follows: *Streptococcus hemolyticus* was dominantly isolated in 4 cases of acute pharyngitis and 1 case of acute tonsillitis and the effective rate was 100 per cent. Capsulated *Hemophilus influenzae* was dominantly isolated in 2 cases of acute tonsillitis and 5 cases of acute bronchitis, and the effective rate was 85.7 per cent; there was one failure in a case of acute bronchitis. *Diplococcus pneumoniae* was dominantly proved in 1 case of acute pharyngitis, 2 of acute tonsillitis, and 6 of acute bronchitis with an effective rate of 88.9 per cent; there was 1 ineffective case in acute bronchitis. As to coagulase-positive *Staphylococcus aureus*, 15 cases of acute pharyngitis (2 cases ineffective), 9 cases of acute tonsillitis (1 case ineffective), and 4 cases of acute bronchitis (1 case ineffective) were dominantly positive and the total effective rate was 85.7 per cent. Except for *Streptococcus viridans*, demethylchlortetracycline was effective against all the other pathogens in more than 80 per cent of the cases. The total effective rate was 75.6 per cent (65 of 86 cases), even when *Str. viridans*, whose pathogenicity is relatively low and which is a persistent bacillus, was added.

Classifying the respiratory tract infections by viral etiology, irrespective of the presence or absence of secondary bacterial infection, tetracycline groups showed a higher rate of cure than the control groups. The rate of cure achieved with tetracyclines, especially in cold-hemagglutinin-positive colds, psittacosis, and hemagglutinating virus of Japan infection, was 80 per cent or more in each group (table V).

Side Effects. The appearance of gastrointestinal disturbances during the course of treatment was as follows: demethylchlortetracycline, 3 (4.0 per cent) of 75 cases; tetracycline sodium citrate mixture, 1 (1.4 per cent) of 73 cases; tetracycline sodium metaphosphate mixture, 5 (3.1 per cent) of 161 cases. These side effects consisted of nausea, soft feces, abdominal pain, etc. None of these necessitated the withdrawal of the drug, and the side effect spontaneously disappeared after completion of the course of treatment. Five patients receiving demethylchlortetracycline, 12 mg./Kg. daily for seven days, and 3 receiving the same drug, 10 mg./Kg. daily for 10 days, exhibited no side effects at all.

While demethylchlortetracycline was given in less cases than the other tetracycline preparations, this drug, administered at a lower dosage than the others, produced about the same effects as the other tetracyclines. For example, 3 children and 2 adults, none of whom had any gastrointestinal disturbance, were given demethylchlortetracycline on an empty stomach at weekly intervals, 15 mg. and 25 mg./Kg. for each child and 450 mg. (three 150 mg. capsules) and 1050 mg. (seven 150 mg. capsules) for each adult, in order to study side effects. After 15 mg./Kg. and 450 mg., nausea and then vomiting occurred after 1½ hours in 1 adult (a 31 year old woman). After 1050 mg., both adults had nausea, and the previously mentioned patient later vomited. After 25 mg./Kg., nausea occurred in all 3 children (a 6 year old girl, an 8 year old boy, and an 11 year old girl), 2 of whom later vomited. The youngest child also had slight abdominal pain. Diarrhea was not observed in any of the patients. However, the gastrointestinal disturbances were transitory: the longest duration was about eight hours' nausea of mild degree in 1 adult (a 31 year old woman). Urine was negative for protein and urobilinogen in all subjects.

These studies show that a dosage of 15 mg./Kg. daily, divided into two doses 12 hours apart, has no appreciable side effect, and that 20 mg. or more in a single dose leads to such gastrointestinal effects as nausea and vomiting.

CONCLUSIONS

The concentration of demethylchlortetracycline in the blood was higher and lasted longer than those of other tetracyclines.

The urinary excretion of demethylchlortetracycline was lower in concentration and appeared later than those of other tetracyclines.

In the treatment of acute pediatric infections, demethylchlortetracycline, 10 to 20 mg./Kg. daily, divided in two doses 12 hours apart, was found as effective as or more effective than the other tetracyclines. This dosage resulted in no appreciable side effects except for gastrointestinal disturbances in a small proportion of the patients treated. Gastrointestinal effects such as nausea and vomiting were frequently observed at higher dosages (more than 20 mg./Kg. in a single dose).

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The Relative Merits of the Four Tetracyclines

L. P. GARROD AND PAMELA M. WATERWORTH

St. Bartholomew's Hospital, London, England

Tetracycline, oxytetracycline, and chlortetracycline have been available for 6, 9, and 11 years, respectively. They resemble one another so closely, both in structure and in antibacterial activity, that it is not easy to define separate spheres of usefulness for them. There are, nevertheless, some differences in the susceptibility to them of certain bacterial species, and these will be redefined here on the basis of personal observations. It has also been said that these three antibiotics possess different degrees of liability to cause gastrointestinal disturbance; this question has been re-examined, and the findings are reported.

A fourth antibiotic has now been added to this group, demethylchlortetracycline; some preliminary observations are reported on the behavior of this substance in vitro and in vivo.

RELATIVE ANTIBACTERIAL ACTIVITY OF THREE TETRACYCLINES

Earlier comparisons of the bacteriostatic activity of these three antibiotics were nearly unanimous in finding three significant differences: greater activity of chlor-tetracycline against staphylococci and streptococci,¹ of oxytetracycline against *Pseudomonas pyocyanea*,² and of tetracycline against *Proteus*.³ All findings, however, were not concordant, and in one extensive study,⁴ the greater activity of oxy-tetracycline against *Pseudomonas* was not confirmed; indeed, in the interpretation placed on these findings, this antibiotic is credited with the lowest instead of the highest activity. These and other less serious discrepancies led one of us to re-examine this question, and the findings have been briefly reported elsewhere.⁵

Method. Strains of bacteria showing abnormal resistance to the tetracycline group were excluded, the object of the study being only to determine which antibiotic had the greatest activity against strains of normal sensitivity. Nine species were included, and 25 strains of each, recently isolated from clinical material, were tested. The antibiotics were incorporated in falling concentrations in plates of ox heart extract peptone agar, pH 7.4, to which blood was added for species requiring it, ruled areas of the plate being inoculated with a 2 mm. loopful of a 1:500 dilution of an overnight broth (or blood broth) culture. This conventional method was adapted to give as close and accurate a comparison as possible by the following precautions: (1) a single large batch of the medium was reserved for the whole series of tests, in order to avoid errors due to varying medium composition; (2) solutions of antibiotics were freshly prepared and plates containing them prepared and inoculated all within one hour; and (3) the plates were read for visible growth after incubation at 37 C. for 18 hours \pm 15 minutes, the main object of each of these measures being to standardize the effect of the instability of chlortetracycline.

Results. The findings for the four gram-positive species are given in a hitherto unpublished form in table I. It is to be remembered that had the period of incu-

TABLE I

Average Minimum Inhibitory Concentrations of 3 Tetracyclines
for 4 Species of Cocci (25 Strains of Each)*

Organism	Chlortetracycline	Oxytetracycline	Tetracycline
<i>Staphylococcus aureus</i>	0.093	0.192	0.144
<i>Streptococcus pyogenes</i> (A)	0.176	0.2	0.183
<i>Streptococcus pneumoniae</i>	0.162	0.342	0.312
<i>Streptococcus faecalis</i>	0.255	0.486	0.356

* Arithmetic mean of minimum inhibitory concentration in $\mu\text{g./ml.}$

bation been longer, the results would have been less favorable to chlortetracycline, but since in therapeutic use its concentration can be maintained, it seems inappropriate to use a form of in vitro test in which the concentration falls, owing to instability, to a very low level before the result is assessed. It is shown that chlortetracycline is the most active of the three antibiotics against all four species of gram-positive cocci, the difference being greatest against the pneumococcus and least against the hemolytic *Streptococcus*.

The findings for gram-negative species are not given here; they are presented in diagrammatic form in the original publication.⁵ They confirmed the greater activity of oxytetracycline against *Ps. pyocyanea* and of tetracycline against *Proteus*. Against *Escherichia coli*, *Klebsiella* species, and *Hemophilus influenzae*, the differences in activity between the three tetracyclines were insignificant.

The differences confirmed here, although only of the order of twofold, are presumably worth taking into account, together with other considerations, in making a therapeutic choice between these drugs.

LIABILITY OF THREE TETRACYCLINES TO CAUSE GASTROINTESTINAL DISTURBANCE

Among several other factors that may rightly influence a choice between these three drugs, their respective tendencies to cause gastrointestinal side effects have been accorded much importance. When tetracycline was introduced, an advantage claimed for it was that this tendency was diminished. In one study⁶ of the frequency of diarrhea, tetracycline was found to cause it least often and oxytetracycline most often, the position of chlortetracycline being intermediate. Such comparisons were not, so far as we are aware, pursued with larger numbers of patients, and the general belief that tetracycline causes fewer side effects than its predecessors is based on impressions rather than on carefully observed facts.

It seemed possible to obtain retrospective information on this matter simply by the study of hospital patients' notes. At this hospital, the number of bowel actions per day is recorded on the patient's chart, and the primary record is kept by conscientious and intelligent nurses. We ascertained from the Statistical Department of the hospital that 355 patients had been treated for pneumonia with one of the tetracyclines in the past 10 years, and the department kindly provided us with the notes of these patients. Patients who died within a few days of admission were

TABLE II

Frequency of Diarrhea in Patients Treated for Pneumonia with Tetracyclines*

Drug	Number of patients	Average daily dose, Gm.	Percentage with diarrhea
Chlortetracycline	59	1.64	25.4
Oxytetracycline	88	1.64	26.1
Tetracycline	141	1.33	22.7

* Defined as more than two bowel actions on any day.

excluded from consideration, as were infants less than 3 months old and patients who had been given morphia, since this is a constipating drug. From the notes of all the remaining patients, the number of bowel actions on seven days, starting with the day on which treatment was begun, were totalled and averaged for each antibiotic. For purposes of comparison, the same calculations were made for 100 patients treated for pneumonia with penicillin.

Results. The findings are presented graphically in figure 1. Some degree of constipation is common in patients confined to bed from any cause, and the relief of this condition, shown by the rise in the penicillin graph, is contributed to largely by the use of aperients. The average daily number of bowel actions increases to a greater extent in patients treated with each of the tetracyclines, but the differences between these are small, although that for tetracycline persists on a slightly lower level. (No explanation can be offered for the greater freedom of bowel movement on day 0 in patients given this antibiotic.)

This slight difference in favor of tetracycline loses much, if not all, of its significance when the findings are presented as in table II. Chlortetracycline was the only one of these antibiotics available in the first part of this 10 year period; it was partly replaced by oxytetracycline, 1951–1954, which in its turn was largely replaced by tetracycline later. In the earlier part of this period larger doses than were thought to be necessary later were often given; the average daily dose of tetracycline

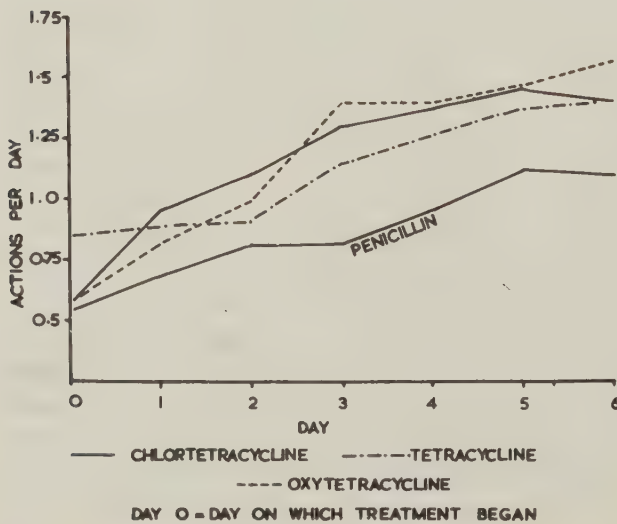


FIG. 1. Average number of bowel actions per day in patients treated for pneumonia with tetracyclines.

TABLE III

Factors by which Sensitivity to Demethylchlortetracycline Exceeds that of Tetracycline

	Factors for six strains	Minimum inhibitory concentration of demethylchlortetracycline, μg./ml.
<i>Staphylococcus aureus</i>	222222	0.3
<i>Streptococcus pyogenes</i> (A)	111111	0.6
<i>Streptococcus pneumoniae</i>	111111	0.6
<i>Streptococcus faecalis</i>	222222	1.2
<i>Bacillus anthracis</i>	122222	0.07
<i>Neisseria gonorrhoeae</i>	2222*	0.15-0.3
<i>Hemophilus influenzae</i>	122222	0.6
<i>Escherichia coli</i>	111111	2.5
<i>Proteus</i> sp.	111222	40-80
<i>Pseudomonas pyocyanea</i>	111211	40-80
<i>Klebsiella</i> sp.	222222	1.2-5.0

* Only four strains tested.

given to adults was 1.33 Gm., whereas that of both chlor- and oxytetracycline was found to be 1.64 Gm. This could fully account for the slightly lesser frequency of diarrhea from tetracycline shown in table II and suggested in figure 1.

This analysis, therefore, does not support the belief that any of the older tetracyclines is more or less liable to cause diarrhea than either of the others.

PRELIMINARY STUDIES OF DEMETHYLCHLORTETRACYCLINE

This new form of tetracycline was first described by McCormick et al.,⁷ and it has several potential advantages over its predecessors; one referred to in this original description is its high degree of stability. Another advantage appears from the pharmacological studies of Kunin and Finland.⁸ Demethylchlortetracycline, dose for dose, attains higher concentrations in the blood than tetracycline, and the concentration persists at a therapeutic level for considerably longer, apparently owing to slower renal excretion. These authors also showed that demethylchlortetracycline is more active than tetracycline against the three bacterial species used in their assays. On the other hand, there is some evidence⁹ that demethylchlortetracycline may have a greater tendency than tetracycline to cause gastrointestinal side effects. Demethylchlortetracycline has been available to us for only a very short time, and the following observations are therefore of a preliminary nature.

In Vitro Activity. The inhibitory activity of demethylchlortetracycline was compared with that of tetracycline by a plate dilution method (twofold dilutions) of the general nature of that described earlier, but without the same rigid standardization of times, since neither of these antibiotics is unstable. The results for six strains each (4 of *Neisseria gonorrhoeae*) of 11 species are stated in table III. In no instance was demethylchlortetracycline less active than tetracycline. Against all or almost all strains of six species, the activity of demethylchlortetracycline exceeded that of tetracycline by twofold, but never more; for each strain of three species, the minimum inhibitory concentrations of both antibiotics were the same, including

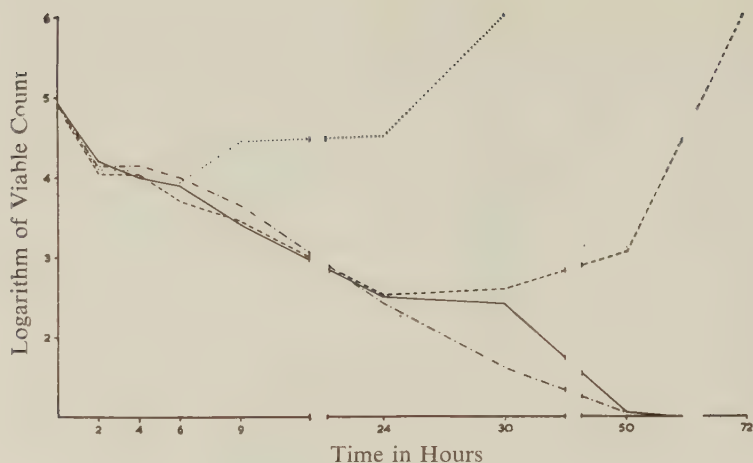


FIG. 2. Viable counts of *Staph. aureus* in broth containing tetracyclines, at 37 C. —, chlortetracycline, 5 µg./ml.; ····, chlortetracycline, 0.5 µg./ml.; —, demethylchlortetracycline, 5 µg./ml.; —·—, demethylchlortetracycline, 0.5 µg./ml.

Streptococcus pyogenes, one strain of which has been reported by others⁸ to be more sensitive to demethylchlortetracycline.

Bactericidal Action. Simple tests of capacity to inhibit growth give an inadequate picture of the action of an antibiotic. A test of the bactericidal action of demethylchlortetracycline, in comparison with that of chlortetracycline, was made as follows.

The test mixtures consisted of 9 ml. broth; 0.5 ml. of freshly prepared antibiotic solution to give final concentrations of 0.5 and 5 µg./ml.; and 0.5 ml. of a 1:1000 dilution of a broth culture of a sensitive strain of *Staphylococcus aureus*. These were placed in a water bath at 37 C., and nutrient agar pour plates from decimal dilutions were prepared at zero time and at intervals thereafter, colonies in these plates being counted after incubation.

These counts are plotted as curves on a logarithmic scale in figure 2. Both concentrations of both antibiotics produced a similar initial fall in the count. The lower concentration of chlortetracycline ceased to be bactericidal after the first six hours, and between hour 24 and hour 30, free growth occurred, the culture becoming turbid. The higher concentration of chlortetracycline continued to be bactericidal up to hour 24, and, after a similar intervening period of slow growth, free growth producing turbidity occurred after hour 50. The action of demethylchlortetracycline, initially indistinguishable from that of chlortetracycline, persisted until the culture was actually sterilized.

These findings reflect the instability of chlortetracycline and the contrasting stability of demethylchlortetracycline, enabling even the low concentration of 0.5 µg./ml. eventually to produce complete sterility. Demethylchlortetracycline may perhaps properly be regarded as simply a stabilized form of chlortetracycline.

Effects of Clinical Administration. Demethylchlortetracycline was administered for periods of 5 to 7 days in doses of 300 or 450 mg., three times a day, to 4 adult patients. In 3 the drug was well tolerated; diarrhea was caused in the last patient, but this was only an exacerbation of a diarrhea previously caused by tetracycline.

The effect of 600 mg. demethylchlortetracycline daily, in two doses of 300 mg. each, given for five days, was compared with that of 1 Gm. of tetracycline given in the same way for the same time, in a crossover study in 48 medical students.

They were given a form to complete on which they entered the number of bowel actions on each day and particulars of any apparent effects of the medication. No serious disturbance of any kind was caused by either antibiotic in any of the subjects. There was no significant difference in the frequency of a mild degree of diarrhea, 10 subjects recording this after taking each drug; the average number of bowel actions per day for the whole period was 1.432 in those taking tetracycline and 1.428 for demethylchlortetracycline.

Among incidental observations made in connection with this trial was cultivation of a specimen of feces passed on the last day of the course and an assay of its antibiotic content by the *Bacillus cereus* cylinder-plate method, using several dilutions of a W/V suspension. Cultivation revealed the frequent presence of *Proteus* and of varying numbers of *Candida albicans*. The presence of *Proteus* did not appear to be correlated with frequency of bowel action, nor was that of *Candida* with the occurrence of pruritus ani, which was noted by only a few of the subjects taking each antibiotic.

Assay of the antibiotic content of the feces yielded the following somewhat surprising results. Tetracycline ranged from 300 to 3500 $\mu\text{g./Gm.}$ (mean, 1196 $\mu\text{g./Gm.}$) and demethylchlortetracycline ranged from 60 to 600 $\mu\text{g./Gm.}$ (mean, 339 $\mu\text{g./Gm.}$).

It appears that a greater proportion of a dose of demethylchlortetracycline than of tetracycline is absorbed, but the residue of both is considerable. (That it is a residue and not the result of biliary excretion we have shown by estimating the antibiotic content of the feces of subjects given tetracycline intramuscularly, and finding little or none.) If means could be found for ensuring more nearly complete absorption of antibiotics of this group, therapeutic effect should be enhanced and intestinal side effects might well be diminished.

SUMMARY

1. The minimum inhibitory concentrations of tetracycline and oxy- and chlortetracycline for certain bacterial species differ; a noteworthy difference is the greater activity of chlortetracycline against some of the gram-positive cocci.

2. These antibiotics have been said to differ in the frequency with which they cause diarrhea. An analysis of records of bowel action of 288 patients treated for pneumonia with the tetracyclines does not confirm that any one of them is more or less liable to cause diarrhea than the others.

3. Demethylchlortetracycline has a higher in vitro activity against some bacterial species than tetracycline. Compared in its bactericidal action with chlortetracycline, it was found to sterilize an inoculum which, in the presence of the same concentration of chlortetracycline, eventually multiplies freely.

4. Demethylchlortetracycline was administered to patients and volunteers and was well tolerated.

ACKNOWLEDGMENT

We are indebted to Lederle Laboratories Ltd. for supplies of demethylchlortetracycline.

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The Susceptibility of *Neisseria gonorrhoeae* to Kanamycin, Leucomycin, Chloramphenicol, and Dextrosulphenidol

JAMES D. THAYER, MIRIAM I. PERRY, FRANCIS W. FIELD,
AND WARFIELD GARSON

Venereal Disease Experimental Laboratory, Venereal Disease Branch, Communicable Disease Center, U. S. Public Health Service, School of Public Health, University of North Carolina, Chapel Hill, N. C.

Untoward reactions to penicillin are becoming more prevalent, and an increasing number of patients with histories of urticaria, angioneurotic edema, or serum-sickness reaction due to previous penicillin therapy are presenting themselves in venereal disease clinics for treatment of their gonococcal infections. The risk involved in re-treating these patients with penicillin has resulted in the use of other antibiotics or of sulfonamides.

Another instance in which therapy other than penicillin is used in the treatment of gonorrhea occurs when the patient fails to respond to two or more courses with clinical and/or bacteriological cure. While the gonococci isolated from such cases are usually found to be of low penicillin susceptibility, they are not "resistant" in terms of blood concentrations that can be developed by adequate therapy. At present the number of such relatively insusceptible strains is around 20 per cent of routine cultures examined.

A study of gonococcus strain susceptibility to new antibiotics or new preparations of established antibiotics may aid in the treatment of gonococcal patients allergic to penicillin or those infected with strains of low susceptibility to penicillin.

Of the four antibiotics reported herein, kanamycin* and chloramphenicol† have received clinical trial for gonorrhea. Dextrosulphenidol‡ is a chloramphenicol analogue in which the *p*-nitro group has been replaced by methylsulfonyl ($\text{CH}_3\text{-SO}_2\text{-}$). Leucomycin is not now commercially available in the United States, but Hata et al¹ have reported it to be inhibitory for *Neisseria*.

There is need for injectable antibiotics other than penicillin for mass treatment and control of venereal disease, and with the appearance of chloramphenicol acid succinate for parenteral administration, new interest in the action of chloramphenicol against strains of the gonococcus has developed.

The antibiotics described in the preceding were tested for their effect on gonococcal susceptibility, bactericidal time, and the development of resistance.

EXPERIMENTAL

Kanamycin, chloramphenicol, and dextrosulphenidol were received as dry crystalline material of 1000 $\mu\text{g./mg.}$ potency; leucomycin potency was 955 $\mu\text{g./mg.}$ Leucomycin, chloramphenicol, and dextrosulphenidol were dissolved in 0.5 ml. ethanol and further diluted in sterile distilled water before adding to proteose no.

* The trade name of Bristol Laboratories for kanamycin is Kantrex.

† The trade name of Parke, Davis & Co. for chloramphenicol is Chloromycetin.

‡ The trade name of The Upjohn Co. for dextrosulphenidol is Propacin.

3 hemoglobin agar (Difco). The final ranges of concentrations used were: 0.1, 0.2, 0.4, 0.8, and 1.0 µg./ml. for leucomycin; 0.12, 0.25, 0.5, and 1.0 µg./ml. for chloramphenicol and dextrosulphenidol; and 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 µg./ml. for kanamycin.

One hundred fifty strains of gonococci were used in the study, of which 25 showed low susceptibility to penicillin (0.10 to 0.225 u./ml.). Suspensions in Trypticase soy broth (BBL) were prepared from agar slant cultures and standardized so that a 3 mm. loopful contained 1000 to 3000 colony-producing units. The surfaces of the agar plates were inoculated by the streak method (8 to 10 streaks/plate). Control plates devoid of antibiotic were streak inoculated with the same cultures for observing maximal growth. After 48 hours' incubation in a candle jar, the plates were observed and the minimal inhibitory concentration taken as the µg./ml. of antibiotic that completely inhibited visible growth.

The time required to sterilize a culture of gonococci exposed in vitro to antibiotic was determined. Fifty ml. of Trypticase soy broth was added to a 250 ml. centrifuge tube containing a Teflon-coated magnetic bar and sterilized at 121 C. for 30 minutes. Antibiotic was then added in an amount twice the minimum inhibitory concentration of the gonococcus strain under test and inoculated with 3.0×10^3 colony-producing units. The antibiotic test and control without antibiotic were placed above a Mag-mix stirrer (adjusted to a slow speed) in the incubator at 35 C. Aliquots of 5 ml. were removed at 0, 12, 24, 36, and 48 hours; centrifuged at 3000 r.p.m. for 15 minutes; and the sedimented organisms washed once and resuspended in 1.0 ml. broth (fivefold concentration). One tenth ml. of the suspension was inoculated on each of five chocolate agar plates and spread with an L-shaped glass rod. After incubation, growth was noted and the number of gonococcus colonies counted.

The ability of gonococci to develop in vitro resistance to the antibiotics under test was studied. Two strains of gonococci were inoculated on chocolate agar slants containing half the minimum inhibitory concentration of antibiotic for the strains (an amount suppressing about 50 per cent of normal growth). The cultures were exposed to antibiotic for about 96 hours and then subcultured to medium containing no antibiotic for 72 hours. This cycle was repeated continually using small increments of antibiotic to induce resistance.

RESULTS

The susceptibility of gonococci to kanamycin, leucomycin, chloramphenicol, and dextrosulphenidol is shown in table I. Seventy per cent of the strains were sensitive to between 4.0 and 8.0 µg./ml. of kanamycin; 95 per cent to from 0.4 to 0.8 µg./ml. of leucomycin; 77 per cent to 0.5 µg./ml. of chloramphenicol; and 88 per cent to 0.25 µg./ml. of dextrosulphenidol. Of 59 strains tested simultaneously against chloramphenicol and dextrosulphenidol, 71 per cent were more sensitive and 29 per cent were of equal sensitivity to the analogue.

Gonococci of low penicillin susceptibility (0.10 to 0.225 u./ml.) were as sensitive to the antibiotics tested as gonococci of high penicillin susceptibility (0.005 to 0.08 u./ml.).

The exposure time required to sterilize a gonococcus culture varied with the strain and particular experiment. Kanamycin, leucomycin, and dextrosulphenidol

sterilized a single strain between 1 and 12 hours. Chloramphenicol sterilized one strain in under 12 hours. However, with another strain 12 to 24 hours were required at one time, while in another experiment with the same strain between 24 and 36 hours were required to effect sterilization.

Increased resistance of two gonococcal strains was induced to kanamycin and leucomycin after 15 weeks on antibiotic-containing medium: from 8.0 to 70.0 µg./ml. or eightfold for kanamycin and from 0.8 to 10.0 µg./ml. or twelvefold for leucomycin. Similar subculturing on chloramphenicol and dextrosulphenidol medium failed to effect more than a twofold change in susceptibility.

DISCUSSION

Ichikawa² reported the successful kanamycin treatment of acute gonorrhea in men with 1 to 2 Gm. daily for a total dose of 1 to 6 Gm. For women 10 Gm. proved necessary. Marmell and Prigot³ found the intramuscular injection of 3 Gm. (1 Gm. three times daily) to be an effective treatment in men, although doses of 2 and 1 Gm. were not entirely effective. Welch et al⁴ have reported average blood levels of kanamycin after injection of a single 1 Gm. dose to have fallen from an initial level of 30.9 to 12.9 µg./ml. by six hours and to 1.6 µg./ml. by 12 hours. Since 30 per cent of the gonococci tested required from 10 to 12 µg./ml. to inhibit growth, it would appear that multiple injections would be necessary to establish and maintain effective blood levels for cure and also to prevent the development of gonococcal resistance to kanamycin.

Little is known of the blood concentrations following the administration of leucomycin. Hata et al¹ found 1.83 µg./ml. four hours after oral administration of 100 to 200 mg. and detectable amounts eight hours after the intravenous injection of 200 mg. Ninety-five per cent of the gonococci tested were sensitive to between 0.4 and 0.8 µg./ml.

Gocke et al⁵ in 1950, and Love and Finland,⁶ in 1954, reported the in vitro susceptibility of gonococci to chloramphenicol. The range of susceptibility found by these authors varied more widely (0.2 to 3.1 µg./ml.) than that reported here (0.25 to 0.50 µg./ml.). This difference may be due to the presence of fresh blood in the medium used by these workers. Enzymatic degradation and serum binding of the chloramphenicol molecule is known to occur.⁷

Oral administration of from 750 mg. to 3 Gm. total dosage of chloramphenicol has proved effective (92 to 96 per cent) for the treatment of acute gonorrhea in men.^{8 10} Clinical trial of chloramphenicol acid succinate has not been reported.

TABLE I
*Susceptibility of Gonococci to Kanamycin, Leucomycin,
Chloramphenicol, and Dextrosulphenidol*

Drug	Number of strains	Susceptibility range, µg./ml.	Average minimum inhibitory concentration µg./ml.
Kanamycin	83	4.0-12.0	8.30
Leucomycin	76	0.2-1.0	0.55*
Chloramphenicol	117	0.25-0.50	0.44
Dextrosulphenidol	73	0.25-0.50	0.28

*Corrected for potency.

Dextrosulphenidol was considerably more active than chloramphenicol; of those strains tested, 88 per cent were sensitive to 0.25 µg./ml. dextrosulphenidol, whereas a similar number (77 per cent) required 0.50 µg./ml. of chloramphenicol to inhibit growth. In human beings, 12 hours after a single oral dose of 1.0 Gm. dextrosulphenidol, an average concentration of 1.6 µg./ml. may be found in the blood.¹¹ This concentration is almost six times the average minimum inhibitory concentration for the strains tested and is present at a time when most strains will have been killed.

Dextrosulphenidol has demonstrated also a remarkable curative effect at low dosage in experimental rabbit syphilis.¹²

Pharmacological studies of dextrosulphenidol reveal certain toxic manifestations. The drug shows significant average decreases in hematocrit and hemoglobin. The average plasma iron (Fe^{59}) binding capacity is also increased over that of chloramphenicol. Further, the in vitro antibacterial spectrum was shown to be inferior to chloramphenicol.¹¹ It is regrettable that these findings have slowed further development of this promising drug as a therapeutic agent.

Such findings should alert us to extreme care in the use of this agent in clinical evaluations; nonetheless, this very promising drug should be carefully studied clinically to evaluate its status as a venereal disease therapeutic agent for use in gonorrhea and syphilis, particularly when the patient is sensitive to penicillin.

SUMMARY

The in vitro susceptibility of gonococci to kanamycin, leucomycin, chloramphenicol, and dextrosulphenidol is reported. The exposure time required by these antibiotics to kill gonococci in vitro was determined, and the development of resistance to the drugs was studied.

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Clinical Trial of Erythromycin Propionate for Acute Anterior Gonococcal Urethritis

MILTON MARMELL, ROBERT F. MORTON, AND AARON PRIGOT

*Departments of Pathology and Surgery of Harlem Hospital,
Department of Hospitals, New York, N. Y.*

Much recent research on chemotherapeutic agents has been motivated by the possibility that increasing the blood levels of an antibacterial substance would increase its therapeutic potential. Clinical confirmation of this hypothesis would be reflected in more rapid inhibition of the invading organism, lower dosages, and shorter courses of medication.

New derivatives of antibiotics have been explored and it has been demonstrated with respect to erythromycin that esters made from two- to four-carbon chain aliphatic acids produce higher serum concentrations than the erythromycin base. The most significant increases were found with the propionyl ester,^{1*} which were attributed to its limited excretion through the biliary tract.

In vitro and animal studies² established an antibacterial range and an order of toxicity essentially the same as those of erythromycin base.

In clinical trials of the new antibiotic, Griffith and his co-workers³ successfully treated infections caused by erythromycin-sensitive organisms. The dosage levels of erythromycin propionate that they employed were 250 or 500 mg. every six hours. Smith and Soderstrom⁴ recently reviewed the biological properties of this antibiotic and described its effectiveness in various infections.

In this paper we report our experiences with erythromycin propionate in the treatment of acute anterior gonococcal urethritis in men.

MATERIAL AND METHODS

Seventy-six men with the diagnosis of gonorrhea established clinically and by smear and culture were treated under the regimens listed in table I. Erythromycin propionate was administered orally in 250 mg. tablets. The criteria of cure were repeated smears and cultures negative for gonococci in a post-treatment observation period. The details of our methods have been described in connection with previous investigations of antibiotics.⁵

RESULTS

Table I presents the results achieved under the various dosage schedules.

Forty-one patients received a total dosage of 8 Gm. of erythromycin propionate in 500 mg. doses, four times daily for four days. Twenty-three patients failed to return for post-treatment observation. Of the 18 who were adequately followed, all were cured.

* The trade name of Eli Lilly & Co. for erythromycin propionate is Ilosone. This investigation was supported, in part, by a grant from Eli Lilly & Co., Indianapolis, Ind.

TABLE I
Erythromycin Propionate in the Treatment of Gonococcal Urethritis

Total dosage and schedule	Number of patients treated	Patients adequately followed	Results	
			Cures	Failures
8 Gm.: 500 mg. 4 times daily for 4 days	41	18	18	0
6 Gm.: 500 mg. 4 times daily for 3 days	29	18	14	4*
3 Gm.: 250 mg. 4 times daily for 3 days	6	6	0	6

* Probable reinfections.

A total of 6 Gm., in 500 mg. doses four times daily for three days, was administered to 29 patients, 11 of whom did not have check-ups for the full period that we consider definitive in gonorrheal infections. Among the 18 whose post-treatment visits permitted the prescribed number of smears and cultures, 14 were cured and 4 were recorded as failures on the basis of positive laboratory findings.

These 4 patients, however, stated that, on completion of the course of medication, they had been free of all clinical symptoms for periods of 16, 15, 8, and 6 days, respectively, after which they had sexual intercourse with the sequel of purulent urethritis. These patients therefore probably represented reinfections.

Of the 11 patients on the 6 Gm. dosage not adequately followed, 3 were examined on one post-treatment visit after the fourth, fifth, and sixth post-treatment days, and all were found symptom free. Although not seen again, these patients were probably cured.

If the number of the probably cured patients and those who were probably reinfected is added to the 14 whose cure was determined with certitude, the ratio of cures to failures on a total dosage of 6 Gm. becomes 21 to 4.

Six patients who were treated with the minimum dosage employed in this study, 3 Gm. of erythromycin propionate, demonstrated no therapeutic benefit on repeated reexamination.

The higher blood levels that other investigators have found with erythromycin propionate do not appear to shorten the course of therapy required for gonorrhea. It is our opinion that the interval of exposure of the gonococcus to antibacterial activity is as important a determinant in the cure of this infection as high antibiotic concentrations.

No untoward side effects or toxicity was encountered in this investigation.

SUMMARY AND CONCLUSION

Erythromycin propionate was administered orally to 76 men with gonorrhea, 42 of whom were adequately observed in the post-treatment period.

All 18 patients who received a total of 8 Gm. of the antibiotic were cured. Fourteen treated with 6 Gm. were cured, and in 4 patients on this dosage, clinical and laboratory evidence of gonorrheal infection persisted. These, however, may be

cases of reinfection rather than drug failure. Three Gm. of the antibiotic failed to cure gonorrhea in 6 patients.

In adequate dosage, the propionate ester of erythromycin as oral medication is safe and effective for the treatment of gonorrhea.

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Further Observations on the Treatment of Gonorrhea in the Male with Intramuscular Kanamycin

MILTON MARMELL AND AARON PRIGOT

*Departments of Pathology and Surgery of Harlem Hospital,
Department of Hospitals, New York, N. Y.*

Reports on the chemistry, pharmacology, and initial clinical investigations of kanamycin,* an antibiotic of Japanese origin, were first presented in 1958. In vitro studies of kanamycin by Gourevitch et al¹ indicated that *Neisseria* are sensitive to kanamycin at concentrations of 2.5 µg./ml., a level easily attainable in body fluids. Ichikawa² used the new antibiotic intramuscularly in the treatment of gonorrhea in both men and women, achieving cures on dosages varying from 1 to 10 Gm. Ruiz Sanchez and his associates³ reported a relapse on the twelfth day in a man who had received 16 Gm. of kanamycin intramuscularly in an eight day period.

In a preliminary study⁴ at this hospital, intramuscular kanamycin was administered to 28 men with acute gonococcal urethritis. Ten patients who received 3 Gm. of the antibiotic were cured. Two Gm. gave less satisfactory results, but we were unable to ascertain whether the failures on this dosage represented relapses or reinfections. One Gm. of kanamycin was unsatisfactory, the cure rate being less than 50 per cent.

Subsequently the study was extended to include 96 additional patients, making a total of 124 patients treated with intramuscular kanamycin under four different dosage schedules as listed in table I.

Diagnostic procedures, methods, and criteria of cure in our clinical trials of kanamycin were previously described.⁵ The patients demonstrated laboratory and clinical evidence of gonorrhea and none had received any medication for the present infection.

RESULTS AND DISCUSSION

Table I presents the results achieved under the different dose schedules. It will be noted that the results in the present series follow rather closely those obtained in the preliminary investigation. The difference in cure rates of 70 per cent and 80 per cent with 2 Gm. of the antibiotic in the preliminary and present series, respectively, is not significant in consideration of the small number of patients involved.

Three Gm. of kanamycin administered in 1 Gm. doses over a period of three days resulted in 25 cures in 25 trials. When this dose was reduced to 1.5 Gm., administered in 0.5 Gm. over a three day period, there were only 2 failures in 56 patients. Thus 1.5 Gm. administered over a period of three days demonstrated

This investigation was supported, in part, by a grant from Bristol Laboratories Inc., Syracuse, N. Y., and the kanamycin was furnished by them.

* The trade name of Bristol Laboratories Inc. for kanamycin is Kantrex.

TABLE I

Kanamycin in the Treatment of Gonorrhea in Men

Total dose, Gm.	Dose schedule	Patients treated and observed	Results	
			Cures	Failures
3	1 Gm. intramuscular on 3 successive days	Previous study, 10	10	0
		Present study, 15	15	0
2	1 Gm. intramuscular on 2 successive days	Previous study, 10	7	3
		Present study, 25	20	5
1.5	0.5 Gm. intramuscular on 3 successive days	Present study, 56	54	2
1.0	1.0 Gm. intramuscular on 1 day	Previous study, 8	3	5

therapeutic efficacy superior to that of 2 Gm. when administered in two days and comparable to that of 3 Gm. when administered over a period of three days. This response to the drug is consistent with the observed rapid excretion of kanamycin in the urine.⁶⁻⁷ The longer period of treatment, even with a lower total dose, allows a longer period of contact between the organisms and the antibiotic and thus a better therapeutic result.

Intramuscular kanamycin was well tolerated by our patients. The brief course of treatment and low total dosage required to eradicate gonorrheal infections are well below the levels of dosage at which toxic reactions to kanamycin are manifested.⁸ No adverse effects attributable to the antibiotic were observed in any of the 124 patients whom we treated with kanamycin.

SUMMARY

Ninety-six additional men patients with laboratory proved gonorrhea were treated with intramuscular kanamycin under various dose schedules, making a total of 124 patients thus treated.

All patients who received 3 Gm. of the antibiotic over a period of three days were cured. Patients treated with 2 Gm. over a two day period gave less satisfactory results. However, 1.5 Gm. of kanamycin administered over a period of three days gave better results than the 2 Gm. regimen and the response was comparable to that achieved with 3 Gm. of the antibiotic.

Since the total dosage for the successful treatment of gonorrhea is well below the levels at which kanamycin toxicity may be encountered, there were no untoward side effects among our patients.

With the increasing incidence of sensitization to penicillin, the availability of another antibiotic for intramuscular administration offers the physician an alternative mode of therapy. In our hands kanamycin was safe and effective for the treatment of acute anterior gonococcal urethritis.

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The Therapeutic Value of Demethylchlortetracycline in Gonorrhea, Lymphogranuloma Venereum, and Donovanosis

MILTON MARMELL AND AARON PRIGOT

*Departments of Pathology and Surgery of Harlem Hospital,
Department of Hospitals, New York, N. Y.*

The effectiveness of the tetracyclines, used singly in their various forms or in combination with other drugs, in the treatment of infections is well established. In 1957, McCormick and associates¹ described a new tetracycline compound, 7-chloro-6-demethylchlortetracycline,* produced from a mutant strain of *Streptomyces aureofaciens* Duggar. Comparative studies by Kunin and Finland² and Sweeney and his co-workers³ demonstrated that this antibiotic produced greater and better-sustained levels of antibacterial activity in human serum after single or multiple doses than did the tetracyclines.

An investigation of demethylchlortetracycline was undertaken to ascertain whether the clinical response in infections encountered in a venereal disease clinic confirmed the therapeutic potential indicated by the superior absorption and prolonged serum concentrations of the new antibiotic. In this paper we report our observations on the effect of demethylchlortetracycline in 81 patients with gonorrhea, 4 patients with lymphogranuloma venereum, and 1 patient with donovanosis.

CLINICAL MATERIAL AND METHODS

The patients with gonorrhea were all men ranging in age from 12 to 76 years, with the mode falling in the 18 to 23 year group. Gonorrhea was established by a clinically manifested purulent urethral discharge showing, on smear, intracellular gram-negative diplococci, which on culture proved to be *Neisseria gonorrhoeae*.

Lymphogranuloma venereum was established in 4 patients. Two had fluctuating, painful inguinal buboes and positive Frei tests. One of the patients, a Negro man 30 years old, had bilateral lymphadenitis and a lesion 3 to 4 cm. in diameter on the lower surface at mid-shaft of the penis. Dark-field examination was negative for *Treponema pallidum*. The lesion was first noted by the patient a week before we examined him and was followed three days later by the involvement of the inguinal nodes. The second patient, a 24 year old Negro man, had an exquisitely painful right inguinal lymphadenitis of two weeks' duration. No penile or other lesion was present when the patient sought clinic treatment, but he gave a history of a penile ulcer nine months earlier. Serological tests for syphilis in both patients were positive.

Two women, aged 41 and 48 years, respectively, had benign rectal stricture,

This investigation was supported, in part, by a grant from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York. Demethylchlortetracycline was furnished for this study by this company.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin.



FIG. 1. Ulcerating granulomatous lesions of donovanosis before therapy.

neoplastic disease being excluded by biopsy. Frei tests were positive in both patients.

Donovanosis was established by the presence of a granulating lesion, in the scrapings of which Donovan bodies were found. The patient with this diagnosis in our series was a 39 year old Negro man who had two adjacent lesions, each 1 cm. in diameter, on the head of the penis (fig. 1). The lesions, which were not painful, had first appeared three weeks previous to examination as itching nodules.

Demethylchlortetracycline was available in 125 and 150 mg. capsules and was administered orally in various dosage schedules as listed in table I.

The patients with gonorrhea were considered cured if post-treatment smears and cultures were negative and remained so for two or more cultures taken during an observation period of not less than six days. Regression of lymphadenitis and com-

TABLE I
Demethylchlortetracycline in the Treatment of Certain Venereal Diseases

Disease	Dosage schedule	Total dosage, Gm.	Number of patients treated	Number of patients followed	Results	
					Cure	Failure
Gonorrhea	250 mg. 4 times daily	1	20	15	14	1*
	250 mg. 3 times daily	0.75	18	15†	15†	—
	150 mg. 4 times daily	0.6	22	17	16	1*
	150 mg. 2 times daily	0.6	21	15‡	15‡	—
Lymphogranuloma venereum. Bubo	150 mg. 4 times daily	7.2	1	1	1	—
Lymphogranuloma venereum. Rectal stricture	150 mg. 4 times daily	3.6	1	1	1	—
Lymphogranuloma venereum. Rectal stricture	150 mg. 4 times daily	18	1	1	1§	—
Donovanosis	150 mg. 2 times daily	18	1	1	1§	—

* Patients admitted to having sexual intercourse during the post-treatment observation period.

† Two patients lost 2 capsules each; hence they received a total dosage of only 300 mg.

‡ One patient was previously treated with 1 Gm. of demethylchlortetracycline.

§ Improvement in perirectal and rectal inflammation with increase in the diameter of the aperture of the stricture. One patient subsequently underwent a Whitehead operation successfully.

plete healing of the lesions were accepted as an indication of the effectiveness of the medication in the buboes of lymphogranuloma venereum and donovanosis.

RESULTS

In table I are presented the results achieved in the treatment of the various diseases under different dosage schedules.

Gonorrhea. Twenty patients with gonorrhea were given 1 Gm. of demethylchlortetracycline orally in four equally divided doses over a period of 24 hours. Five failed to report for post-treatment observation. Of the 15 adequately observed, 14 were cured, and 1 returned on the ninth post-treatment day with a purulent urethral discharge, which was positive for gonococci. He admitted sexual indulgence three days previously, when he believed himself cured because of disappearance of the original clinical symptoms. Among the 14 cured patients were 6 who were seen in the clinic for other reasons three months after treatment for this gonorrheal infection. All had remained symptom free during that interval.

Eighteen patients received a total of 750 mg. of the drug in three equally divided doses of 250 mg. over a period of 24 hours. Three of these patients did not return to the clinic. All of the 15 who had post-treatment examinations were cured. Among them was 1 man who had been cured of a previous gonococcal urethritis with 1 Gm. of demethylchlortetracycline.

Forty-three patients were treated with a total of 600 mg. of demethylchlortetracycline and of these 11 did not report for further observation. Of those who were adequately followed, 17 received the antibiotic in 150 mg. doses four times a day for one day, and 15 received the drug in 150 mg. doses two times a day over a period of two days. Cultures from one patient who admitted to intercourse during the observation period remained positive and this case is considered a reinfection. All others responded favorably to demethylchlortetracycline.

Among the patients on a prescribed dosage of 150 mg. twice daily for two days were 2 patients each of whom lost two of the four capsules they had been given. These patients therefore ingested only half of the prescribed medication, the 300 mg. taken on the first day. However, the urethral discharge in these patients had disappeared completely on the fourth post-treatment day and cultures of urethral swabbings were negative for gonococci.

Although in all the dosage schedules studied, the ultimate result was the cure of the patient, it was noted that the greater the dosage, the sooner the disappearance of the residual watery discharge observed in follow-up examinations. From the standpoint of this residual discharge, the two day treatment with a total of 600 mg. appeared more effective than the same quantity of medication taken in one day.

Lymphogranuloma Venereum. Two men with buboes of lymphogranuloma venereum were treated with demethylchlortetracycline. Although the patient with the penile ulcer had a negative dark-field examination, he was treated with penicillin because of the highly positive serological test. He received a total of 1.2 billion units, 1.2 million units per day for 10 days, with only minor influence on the ulceration. Such response as was observed we attribute to the effect of the penicillin on secondary contaminating organisms.

Dosage of demethylchlortetracycline for both patients with lymphogranuloma



FIG. 2. Cicatricial formation at conclusion of treatment totalling 7.5 Gm. of demethylchlortetracycline.

was 150 mg. ingested 4 times a day. The patient with the penile lesion received the drug for 12 days for a total of 7.2 Gm. The second patient was treated for six days and thus received a total of 3.6 Gm. All clinical symptoms disappeared in both patients.

The 2 women with benign rectal strictures secondary to lymphogranuloma venereum were treated with 150 mg. of the drug four times daily for 30 days. Both patients improved with considerable reduction in the rectal tenesmus and discomfort. Repeated proctoscopic examinations showed progressive reduction in the inflammatory reaction with an increase in the aperture of the rectal stricture. One patient had a successful Whitehead operation. Demethylchlortetracycline therapy was continued during the postoperative period and the patient made an uneventful recovery. The second patient is awaiting operation for the rectal stricture.

Despite the prolonged course of treatment in these cases, no side effects or toxic reactions to demethylchlortetracycline were found in the hepatic, cardiovascular, or renal systems.

Donovanosis. The patient with donovanosis received demethylchlortetracycline in 150 mg. doses twice daily. The lesion showed definite signs of healing in eight days and was completely healed after he had taken 7.5 Gm. of the antibiotic (fig. 2).

Toxicity. No untoward reactions were reported by any of our patients even when questioned specifically regarding side effects.

DISCUSSION

In this clinical trial we found demethylchlortetracycline an efficient therapeutic agent for gonorrhea, lymphogranuloma, and donovanosis. It is not the purpose of this paper to compare the potential of this antibiotic with that of similar drugs. The pitfalls of such comparisons have been discussed by Finland.⁴ Striking, however, and noteworthy is the small total dosage that was required to cure our patients. The oral dosage of demethylchlortetracycline for the cure of gonorrhea, for instance, approaches that reported for the tetracyclines administered intramuscularly.^{5,6}

In patients on a twice daily dosage schedule, the clinical response was as prompt and favorable as in those on a more frequent regimen. It will also be noted that 2

patients with gonorrhea for whom a two day twice daily program was prescribed lost half of their medication. Nevertheless, they responded favorably to the 300 mg. that they did take. Hence it appears that lower dosages of demethylchlortetracycline than were employed in this investigation may be effective for gonorrhea.

SUMMARY AND CONCLUSION

Demethylchlortetracycline was used in the treatment of 81 men with gonorrhea, 2 men with lymphogranulomatous buboes, 2 women with rectal strictures due to lymphogranuloma infection, and 1 man with donovanosis.

Sixty-two of the gonorrhea patients returned for the specified number of follow-up examinations. With the exception of 2, all were cured with the dosage schedules employed. The 2 patients in whom positive cultures were recorded in the post-treatment interval probably represented reinfections.

The lowest dosage of the antibiotic resulting in the cure of gonorrhea was 300 mg.

Inguinal involvement and buboes in 2 men with lymphogranuloma and 1 with donovanosis responded favorably to demethylchlortetracycline and the patients were discharged from treatment in periods ranging from 13 to 25 days.

In 2 women with lymphogranulomatous strictures, rectal inflammation subsided and the lumen of the constricted area increased in diameter in the course of 30 days' therapy during which each patient received 18 Gm. of the drug. One patient has had surgery for removal of the stricture and the other is awaiting operation.

No toxicity or untoward reactions of any kind occurred in our patients.

We found demethylchlortetracycline an excellent drug for the treatment of gonorrhea, lymphogranuloma venereum, and donovanosis.

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Injectable Tetracycline in Gonorrhea

S. M. LAIRD

St. Luke's Clinic, Manchester, England

Mounting evidence of diminished sensitivity of some strains of *Neisseria gonorrhoeae* to penicillin^{2, 3, 5} has refocussed attention on the efficacy of other antibiotics in gonorrhea. Streptomycin resistance has already been described^{1, 4} and further study of the tetracyclines in gonorrhea seemed desirable. The venereologist, in Britain at least, has a strong antipathy to oral therapy, since many of his patients cannot be relied upon to carry out self-medication conscientiously. The opportunity of conducting a clinical trial of an injectable preparation of tetracycline phosphate* was therefore welcome.

PRESENT INVESTIGATION

Thirty-three gonococcal infections in 31 unselected men have been treated with injectable tetracycline at St. Luke's Clinic. In each case acute gonorrhea was diagnosed by microscopic examination of Gram-stained smears of urethral pus; routine serum tests for syphilis were negative. Of the 31 patients, 20 (64 per cent) were single, 6 (20 per cent) were married, and 5 (16 per cent) were widowers, divorced, or legally separated. Their mean age was 29 years, the youngest was 17 years and the oldest 58 years. Twenty-one patients were white and 10 were Negroes from British West Indies or West Africa. Ten (thirty-two per cent) denied previous venereal disease and 21 (68 per cent) admitted, or were known to have had, one or more previous attacks of urethritis.

Two dosage schedules were used; 20 infections were treated with a single, intramuscular injection of tetracycline phosphate (equivalent to 250 mg. tetracycline hydrochloride), and 13 patients were given a similar injection on two consecutive days. Patients were re-examined 48 hours after injection and thereafter at weekly intervals, special attention being given to evidence of urethral infection, the injection site, and the patient's subjective reaction to injection. Follow-up studies averaged 44 days for those given one injection and 23 days for those receiving two injections.

RESULTS

Eight patients did not re-attend after injection and the outcome is uncertain but clinical cure probably resulted (table I). Recurrences occurred in 13 infections treated with one injection; 5 of these were probable failures but in the remaining 8 re-infection was almost certain. Of those receiving two injections, recurrence occurred in 2 of which 1 was probably due to re-infection. Thus, with only one injection there were 5 failures (31 per cent) in 16 patients followed and, with two injections, 1 failure (11 per cent) in 9 patients followed. If the 4 patients in each

* The trade name of The Upjohn Co. for tetracycline phosphate is Panmycin phosphate.

TABLE I

Findings and Results in 31 Men Treated for 33 Attacks of Gonorrhea

	Treatment	
	1 injection	2 injections
Number of patients		
White	13	8
Negro	5	5
Total	18	13
Number of infections treated	20	13
Failed to re-attend after treatment	4	4
Recurrence of gonorrhea	13	2
Probable re-infection	8	1
Probable failure	5 (25%)	1 (8%)

group who did not attend after treatment are accepted as cures, as is probable, the failure rates become 25 per cent with one injection and 8 per cent with two injections.

No systemic ill-effects occurred and no local disturbance was noted objectively at the site of injection. The threshold of discomfort or pain varies so much from one individual to another that only an approximate assessment could be made, but 7 patients (24 per cent) had no local discomfort, 6 (19 per cent) had mild discomfort, 7 (24 per cent) had severe discomfort, and 10 (32 per cent) had severe pain for 6 to 24 hours after injection. Those patients who had previously experienced injections of procaine penicillin thought that the injectable tetracycline caused much more discomfort. No racial difference in subjective response emerged.

SUMMARY AND CONCLUSIONS

The efficacy of an injectable tetracycline phosphate complex has been assessed in 33 men with gonorrhea. In a dosage equivalent to 250 mg. of tetracycline hydrochloride daily for two days, it appeared to cure about 90 per cent of patients. While local discomfort occurred in more than half the patients, the absence of any systemic side effects provides an advantage over the oral use of tetracyclines. Although penicillin remains the treatment of choice for gonorrhea, this injectable preparation of tetracycline phosphate would be a suitable alternative in penicillin-resistant cases.

ACKNOWLEDGMENT

I am indebted to The Upjohn Company, Kalamazoo, Mich., for a supply of Panmycin phosphate.

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The Use of Injectable Tetracycline Preparations in the Treatment of Gonorrhea

R. R. WILLCOX

St. Mary's Hospital, London, England

Two developments have increased the need of a tetracycline preparation, both effective and well tolerated, which can be given intramuscularly. One is the increasing frequency of severe allergic reactions to penicillin, which necessitates an alternative drug, both for patients with syphilis and for the increasing numbers of patients who have repeated attacks of gonorrhea who are sensitive to penicillin. The second development is the occurrence in some areas of increasing failure rates in gonorrhea with doses and preparations of penicillin, which were at one time more effective. The need of having a good injectable alternative to penicillin to be held in reserve is obvious.

The present paper concerns the use of two injectable tetracycline preparations in the treatment of uncomplicated gonorrhea—tetracycline phosphate* with lidocaine and a new water-soluble pyrrolidinomethyl tetracycline compound with lidocaine.

The tetracycline phosphate was supplied in single doses, each equivalent to 250 mg. of tetracycline hydrochloride. The yellow powder was dissolved in 2 ml. of glycerol acetal diluent supplied in sterile ampoules for each dose. When 500 mg. was given, two such doses were given as a single intramuscular injection. The pyrrolidinomethyl tetracycline compound was supplied as a white powder, the dry fill also being equivalent to 250 mg. of the hydrochloride. Each dose was dissolved in 1.7 ml. of distilled water.

CASE MATERIAL

One hundred twelve men with acute uncomplicated gonorrhea were treated with intramuscular tetracycline. Fifty-seven of the patients were Negroes, with 53 from the West Indies, 3 from West Africa, and 1 from Uganda, Central Africa. Of the remaining 55, 28 were from the United Kingdom, 11 from Eire, 3 from Hungary, 2 each from Pakistan, South Africa, and the United States, and 1 each from Greece, India, Italy, Malta, Poland, South America, and Spain. The average age for the Negro men was 27.4 years (range 18 to 46), and for the others 25.9 (range 18 to 66). Nineteen were married and 93 were single.

Only 39 patients (14 Negroes) had had no previous venereal disease, but the remainder had had 268 previous attacks of gonorrhea, 57 of nongonococcal urethritis, 5 of anxiety concerning venereal disease, 3 of syphilis, 3 of genital sore, and 1 each of condyloma acuminatum molluscum contagiosum, paraphimosis, penile thrombosis, epididymitis, penile boil, urethral stricture, and scabies and tinea causing suspicion of venereal disease—a total of no less than 345 previous attacks. The 57 Negro patients had had 233 previous attacks (average 4.1), and the 55 white patients had had 112 previous attacks (average 2.0). Some of the Negro

* The trade name of The Upjohn Co. for tetracycline phosphate is Panmycin.

TABLE I

Follow-Up and Results in 112 Cases of Gonorrhea Treated with 250 to 500 mg. of Intramuscular Tetracycline

Follow-up	No. patients followed			Results			Non-gonococcal infection
	Total	White	Negro	Satisfactory	Failure	Reinfection	
0	112	55	57	—	—	—	—
1-3 days	87	45	42	16	3	—	1
4-7 days	67	31	36	12	10	—	—
8-14 days	45	23	22	10	7	3	2
15-21 days	23	9	14	8	—	—	1
22-28 days	14	5	9	4	2	—	—
1-2 months	8	3	5	3	—	4	—
2-3 months	1		1	—	—	1	—
Total	87			53	22	8	4
Per cent	100			60.9	25.3	9.2	4.6

patients had been particularly promiscuous, one having had 32, another 21, and 4 others between 11 and 19 previous attacks each.

The discharge had been present before treatment for one to three days in 78 cases, for four to seven days in 28, for 8 to 14 days in 4, for 22 to 28 days in 1, and for more than one month in 1. Dysuria was noted by 83 patients and noted by 29. The disease had apparently been caught from a stranger (including prostitutes) by 73 patients, from a friend by 34, from a man by 2, and from the wife by 1; 2 persons denied exposure. The apparent incubation period was one to three days in 46 cases, four to seven days in 39, 8 to 14 days in 11, 15 to 21 days in 6, 22 to 28 days in 1, and more than 28 days in 7; 2 patients denied sexual relations.

In all cases gonococci were found by urethral smear before treatment. The Wassermann and V.D.R.L. (or Kahn) reactions were both negative before treatment in 99 patients; the Wassermann reaction was negative and the V.D.R.L. reaction was positive in 12 patients (9 of whom were in West Indians and 1 in a West African Negro), and in 1 case the serum was too hemolyzed for testing. The gonococcal complement fixation test was performed on sera from 66 patients. The result was negative in 65 cases and positive in 1 West Indian patient.

CASE MANAGEMENT

Twenty-two patients were given single intramuscular injections of 250 mg. of tetracycline phosphate with lidocaine, 66 were given 500 mg. of the same preparation, and 24 received single intramuscular injections of 250 mg. of a pyrrolidino-methyl tetracycline compound with lidocaine, which was water soluble.

Following treatment, the intention was to see patients after two to three days and again, 1, 2, 4, 8, and 12 weeks from treatment. Follow-up was terminated in the event of failure, reinfection, or a subsequent nongonococcal infection. Not all patients attended at the times instructed, and a full three months of follow-up has not been possible so far in all cases. In all but 5 of the 112 cases (treated with

TABLE II

Acute Gonorrhea Treated with Intramuscular Tetracycline: Comparison of 3 Schedules

Schedule	No. treated	No. followed	Satisfactory	Non-gonococcal infection	Reinfection	Failure	Per cent failure of those followed
250 mg. tetracycline phosphate	22	19	7	1	3	8	42.1
500 mg. tetracycline phosphate	66	48	32	3	5	8	16.7
250 mg. pyrrolidinomethyl-tetracycline	24	20	14	—	—	6	30.0
Total	112	87	53	4	8	22	—
Per cent	—	100	60.9	4.6	9.2	25.3	Av. 25.3

500 mg. of tetracycline phosphate with lidocaine) a full month has elapsed since treatment before presenting this report.

At each visit the urethra was examined for discharge (a smear being taken if present) and the urine for haze and threads. It was planned to make two examinations of the prostatic fluid during surveillance and to make final serum tests for syphilis at three months. No satisfactory criteria exist to distinguish relapse or reinfection. A history or denial of further sexual exposure, as the only means available, was used in this series.

TABLE III

Results in White and Negro Patients

Schedule	No. treated	No. followed	Non-gonococcal infection	Reinfection	Failure	Per cent failing
White Patients						
250 mg. tetracycline phosphate	14	12	—	1	6	50.0
500 mg. tetracycline phosphate	31	23	3	2	4	17.4
250 mg. pyrrolidinomethyl tetracycline	10	9	—	—	2	22.2
Total	55	44	3	3	12	Av. 27.3
Negro Patients						
250 mg. tetracycline phosphate	8	7	1	2	2	28.6
500 mg. tetracycline phosphate	35	25	—	3	4	16.0
250 mg. pyrrolidinomethyl tetracycline	14	11	—	—	4	36.4
Total	57	43	1	5	10	Av. 23.3

RESULTS

The over-all follow-up and results are shown in table I.

Of 112 patients treated, 87 were followed; there were 22 failures (25.3 per cent) plus 8 suspected reinfections (9.2 per cent). The results obtained with the three schedules are compared in table II.

The results with single injections of 250 mg. tetracycline phosphate were bad (42.1 per cent failures), but vastly improved results (only 16.7 per cent failures) were obtained when 500 mg. was given. Improved results (30.0 per cent of failures), compared with the same dose of tetracycline phosphate, were obtained when the 250 mg. equivalent of pyrrolidinomethyl tetracycline was used.

The results in white and Negro patients are contrasted in table III; these results show nothing to indicate that the over-all failure rate was larger in the Negro than in the white patients, in spite of the former group, as judged by previous history of venereal disease, being the more promiscuous group.

SIDE EFFECTS

A disadvantage of tetracycline preparations so far available for intramuscular injection has been local discomfort or pain produced by injection. Although some patients may regard this as indicative of "strong medicine," it has prevented the use of dosage schedules involving single injections of more than 500 mg.

In the present series of 22 patients given single injections of 250 mg. of tetracycline phosphate with lidocaine, information regarding local tolerance is available in 17 cases. Ignoring minor sensations, local discomfort or pain was reported by 11 of these (64.7 per cent). Of the 66 patients given 500 mg. of the same preparation, information regarding local tolerance is available in 47 cases. Local discomfort or pain was reported in 28 cases (59.6 per cent). Of the 24 patients given 250 mg. of pyrrolidinomethyl tetracycline with lidocaine, information is available in 20 cases, of whom 11 reported some local discomfort or pain (55 per cent). In the latter series, however, the duration of discomfort was markedly less. In only 4 cases (20 per cent) did the duration exceed four hours. On the other hand, of the 64 patients treated with 250 or 500 mg. of tetracycline with lidocaine in glycerol acetal on whom information is available, the duration of the local discomfort or pain exceeded four hours in 35 cases (54.7 per cent). In all cases the local discomfort or pain passed off without tissue necrosis or other complication.

Of systemic side effects, 2 patients felt slightly faint following 500 mg. of tetracycline phosphate with lidocaine. This was associated with local pain at the injection site and the faintness soon passed off. One patient fainted immediately on having a similar injection but rapidly recovered. Local pain was not complained of in this case, and the reaction was believed to be due to needle puncture rather than to any substance injected. One case of anaphylactoid reaction, with fall in blood pressure, was, however, noted with the same preparation. Recovery was prompt following an injection of adrenaline hydrochloride, and it is not known whether the tetracycline phosphate or the lidocaine was responsible. No systemic side effects were noted in the patients receiving the pyrrolidinomethyl tetracycline compound.

SUMMARY AND CONCLUSIONS

1. The need for a tetracycline preparation that can cure gonorrhea in a single injection is stressed.

2. One hundred twelve men with acute gonorrhea were treated with single intramuscular injections of one of two tetracycline preparations. Of 22 patients given 250 mg. hydrochloride equivalent of tetracycline phosphate with lidocaine in glycerol acetal, 19 were followed; there were 8 failures (42.1 per cent). Of 66 patients given single injections of 500 mg. of the same preparation, 48 were followed; there were 8 failures (16.7 per cent). Of 24 patients given 250 mg. hydrochloride equivalent of a new, water-soluble preparation of pyrrolidinomethyl tetracycline, 20 were followed; there were 6 failures (30.0 per cent).

3. Tetracycline with lidocaine in single injections of 250 mg. thus gave bad results in the treatment of gonorrhea. Vastly improved results (83.3 per cent success) were obtained when the dosage was increased to 500 mg., and such a dosage can reasonably be recommended for patients with gonorrhea who are allergic to penicillin.

4. An increase of dosage or the use of multiple injections of this preparation is, to a considerable extent, precluded by the local discomfort or pain experienced by more than half of patients.

ACKNOWLEDGMENTS

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Intramuscular Oxytetracycline in the Treatment of Acute Gonococcal Urethritis

ELMER H. LOUGHLIN AND LOUVERTURE ALCINDOR

Faculty of Medicine, Port-au-Prince, Haiti

In the mass treatment of venereal disease, particularly acute gonorrhea, repository penicillin generally has been considered as the superior antibiotic. None of the newer antibiotics yet has superseded repository penicillin, especially when such facts as route and facility of dosage, local adverse reactions, and cost per treatment are considered.

Although sensitivity of gonococci to penicillin extends from 0.0002 to 0.333 units/ml., it has been signally fortunate that neither absolute nor increasing resistance has been acquired by this organism. On the other hand, although accurate figures generally are not available in medical literature, it has been recognized that allergic reactions to penicillin are occurring with increasing frequency. In spot surveys conducted during 1952 and 1953 in 11 cities with populations of 100,000 or more, Welch et al¹ found 88 cases of anaphylactic shock, 28 per cent of which were fatal. In a nationwide survey from 1953 to 1957, Welch et al² determined that intramuscular injections of penicillin caused anaphylactic shock in 611 cases with 63 deaths; also, that from 1953 to 1956, there was a definite increase in the number of reactions with 301 occurring during the latter year. Although by contrast, anaphylactoid reactions were encountered only with extreme rarity following intramuscular injections of the broad-spectrum antibiotics, it is appreciated that there was considerable disparity between the number of injections of penicillin and of broad-spectrum antibiotics. Nevertheless, in the mass treatment of gonorrhea, there is a definite need for other antibiotics that can be as effectively and as easily administered as repository penicillin.

To date, the broad-spectrum antibiotics when administered intramuscularly either have proved to be less effective, or have caused too much local pain when given in adequate single doses. Another significant disadvantage of the intramuscular dosage forms of the broad-spectrum antibiotics, particularly the tetracyclines, has been the fact that they consisted of dry powders that had to be freshly dissolved in water or solutions of local anesthetics before administration. Recently, Hammer et al³ reported the use of an intramuscular dosage of a "preconstituted solution" or oxytetracycline prepared in 50 per cent aqueous N,N-dimethylacetamide and containing 2 per cent of lidocaine, a local anesthetic. Although moderate immediate pain and delayed local pain occurred after intramuscular injections of 100 mg. of this oxytetracycline, and "some tenderness to palpation or mild induration" was encountered in 2 of 4 persons receiving four or more doses, none of these was considered to be significant. Also, they found that adequate antibiotic levels in the blood could be attained with repeated doses of 100 mg. of oxytetracycline administered intramuscularly at intervals of 12 hours.

The present studies were made to determine the tolerance to and the clinical

effectiveness of intramuscular oxytetracycline, 125 mg./ml., with 2 per cent lidocaine in 80 per cent aqueous propylene glycol solution in the treatment of acute gonococcal urethritis.

METHODS AND MATERIALS

One hundred consecutive men with acute gonorrhea were treated with this formulation of oxytetracycline. Two ml., containing 250 mg. of oxytetracycline were administered intramuscularly into each buttock, the total single dose being 4 ml. containing 500 mg. of oxytetracycline.

The diagnosis of gonorrhea was determined by a history of exposure, local physical signs, and the finding of gonococci in Gram-stained spreads of urethral exudate. Only patients with the acute stage of gonorrhea were selected for evaluating this oxytetracycline dosage.

The patients were questioned regarding any subjective manifestations immediately after the injections, within one hour, subsequently at 24 and 72 hours, and again on the seventh day post-treatment. At these times, the sites of injection were examined by palpation for possible tenderness and induration, as well as swelling and warmth. Also, at these times, spreads and cultures of urethral exudate or discharge, if any were present, were made and examined for gonococci. When bacteriological examinations were negative during this post-treatment period and the clinical manifestations of infection had disappeared, cure was thought to have been accomplished.

RESULTS

Tolerance. Twenty-eight patients complained of immediate slight burning discomfort after injection of this formulation of oxytetracycline in one or the other of the buttocks. There was no significant residual pain, but in 15 of those who experienced immediate discomfort, there was local soreness while walking during the first day or two after injection. There was no appreciable delayed tenderness, no determinable swelling or induration, and no limitation of motion resulting from these injections.

Most of these patients recalled having received injections of penicillin, probably repository, at various times during the past 10 years. None of them considered that these injections of this formulation of oxytetracycline caused any more discomfort.

Effectiveness. Using the afore-mentioned criteria for determining the results of treatment with the previously mentioned dosage of oxytetracycline, 93 of the 100 patients were considered to have been cured of acute gonorrhea with the single bilateral intramuscular dose of 500 mg. Almost invariably, the symptoms of acute urethritis, including the purulent discharge, abated and vanished within 12 hours. In about one third of the cases, a slight quantity of mucoid material, which was bacteriologically negative, could be expressed for a day or two. The 7 patients who relapsed, although reinfection was probable in some, responded to a second dose of 500 mg. of oxytetracycline intramuscularly.

Toxic Reactions. None of these patients, who received this dosage of intra-

muscular oxytetracycline manifested any systemic adverse reactions. No allergic reactions were encountered.

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Preliminary Report on the Effect of Dextrosulphenidol in Experimental Syphilis

WARFIELD GARSON, THOMAS C. WASHBURN, AND JOHN W. CLARK, JR.

Venereal Disease Experimental Laboratory, Venereal Disease Branch, Communicable Disease Center, Public Health Service, and School of Public Health, University of North Carolina, Chapel Hill, N. C.

Syphilis is still a significant public health problem; in 1958 it ranked fourth among all reported communicable diseases in the United States. Although penicillin remains the drug of choice for treatment, hypersensitive reactions to penicillin do occur, and their ever-present danger necessitates a continuing search for other antibiotics that are effective in curing syphilis. Such investigations have been made in this laboratory by Garson and McLeod^{1,2} and Washburn et al.³ in their studies of the effect of novobiocin and kanamycin on experimental syphilis in rabbits. Intramuscular forms of drugs are evaluated for syphilitic therapy of clinic patients, whose reliability in taking oral medication is often questionable.

This study utilized dextrosulphenidol,* the methylsulfonyl analogue of chloramphenicol. Chloramphenicol itself has been shown to have a curative effect on syphilis in man⁴ and rabbits⁵ at total dosage levels of 12 to 24 Gm. and 800 mg./Kg. respectively. Dextrosulphenidol produces less acute toxicity in animals and higher levels of excreted active drug than does chloramphenicol. These facts have led to the hope that dextrosulphenidol may cure syphilis at lower dosages than does chloramphenicol.

EXPERIMENTAL METHODS

This study consisted of treating rabbits having early cutaneous syphilis with dextrosulphenidol in a manner similar to that described by Garson and McLeod¹ and Washburn et al.³ Young adult male rabbits of mixed breeds were infected intracutaneously at six sites on the back with a suspension of *Treponema pallidum* (Nichols strain). The suspension of treponemes was obtained by mincing and emulsifying testes from rabbits with early syphilitic orchitis. The emulsified testes were added to a mixture of 50 per cent normal rabbit serum in physiological saline and shaken. This mixture was centrifuged at 500 G. for 10 minutes to remove the gross particles of testicular tissue. The supernatant contained the treponemes at a concentration of about 2×10^7 treponemes/ml., as determined by count on a calibrated dark-field microscope. The rabbits were infected with 0.2 ml./site of this suspension.

At most of the sites where treponemes had been injected, macular lesions appeared after 48 hours. Syphilitic papules developed rapidly in size to a mean diameter of 10 mm. by six days after infection. Fluid for observation of treponemes

* The trade name of The Upjohn Co. for dextrosulphenidol is Propacin. The drug was supplied for this study by this firm.

TABLE I
*Comparison of Dextrosulphenidol and Chloramphenicol in Early
 Syphilis of Rabbits;
 Time of Conversion of Lesions to Dark-field Negativity*

Total dosage, mg./Kg./day	Days from start of treatment	
	Dextrosulphenidol	Chloramphenicol
Control	No conversion	No conversion
0.89	11	
1.8	7	
3.6	6	
7.1	4½	39
14.3	4	8
28.6	3½	6
57.1	3	5
114	3½	3

by dark-field microscopy was removed from the periphery of each papule. At six days after infection all lesions were dark-field positive. Intramuscular administration of dextrosulphenidol was then given every 12 hours for one week. The dosage levels ranged from 0.89 to 114 mg./Kg./day in two-power steps, with 5 rabbits at each level (a range of total dosage from 6.25 to 800 mg./Kg.). A control group of 5 rabbits was infected but not treated with the antibiotic.

Evaluation of the response to dextrosulphenidol was made clinically and serologically. The course of the syphilitic infection was followed by periodically describing and measuring the diameter of the cutaneous lesions. Fluid from one lesion/rabbit was subjected to dark-field examination each day when the lesions were measured. Close observation of the back and of the testes was made to date the occurrence of dark-field-positive secondary syphilitic lesions.

The serological response to syphilitic infection and treatment was followed by testing serum obtained before infection and at intervals after treatment was started. The serological tests used were the VDRL slide test⁶ and the tpcf 50 test.^{7*}

The general condition of the rabbits was observed, weights were measured at intervals, and autopsies were performed on rabbits that died during the experiment.

The final decision in regard to biological cure will be made after performing transfers of popliteal lymph nodes to nonsyphilitic rabbits in the usual manner.³

RESULTS

The effects of dextrosulphenidol on the clinical course of syphilis in rabbits are shown in tables I and II and figure 1. Figure 1 represents the time course of the mean diameters of cutaneous lesions. The usual progression and regression of untreated syphilis over a 60 day period is shown by the control group. The group at 0.89 mg./Kg./day started to show regression of the lesions; however, relapses began to occur at 20 days after the start of therapy. Similarly, part of the group at 1.8 mg./Kg./day began to relapse at about 30 days, and the size of the lesions increased. The groups at the six highest levels of treatment had a rapid regression

* Tests were performed by Dr. J. Portnoy's section.

TABLE II

*Comparison of Dextrosulphenidol and Chloramphenicol in Early Syphilis of Rabbits:
Evidence of Clinical Relapse*

Total dosage, mg./Kg./day	Dextrosulphenidol		Chloramphenicol	
	No. of relapses	Days from start of treatment to relapse	No. of relapses	Days from start of treatment to relapse
Control	5/5	Primary lesions remained	5/5	Primary lesions remained
0.89	5/5	31		
1.8	2/5	36		
3.6	0	—		
7.1	0	—	5/5	25
14.3	0	—	4/5	34
28.6	0	—	1/5	27
57.1	0	—	1/5	41
114	0	—	0	—

to a healed state without relapses. For graphic purposes, the six similar groups were combined into two curves.

The rate of conversion of cutaneous lesions to dark-field negativity after the drug was started is shown in table I. The controls retained their dark-field-positive lesions for about 47 days. The groups at the three highest levels of therapy be-

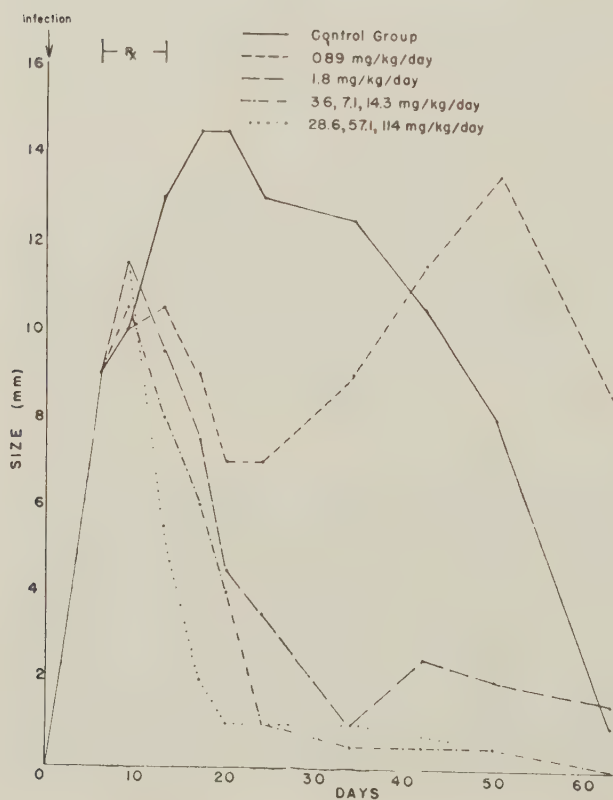


FIG. 1. Effect of dextrosulphenidol in early syphilis of rabbits: Mean diameters of cutaneous lesions.

came dark-field negative at about the third day after the start of the drug. The other groups had slightly slower rates of conversion.

Data on clinical relapses are shown in table II. All of the controls had long-lasting primary lesions. All rabbits in the group on 0.89 mg./Kg./day had dark-field-positive cutaneous relapses at about 31 days after the start of the drug. Two of the 5 rabbits in the group at 1.8 mg./Kg./day relapsed at 37 days. All other treated rabbits remained clinically cured.

The serological course of this experiment is shown in figures 2 and 3. The similar trends of the groups at the six highest levels of drug allowed them to be combined into two larger groups for graphing purposes. Results with the VDRL slide test are shown in figure 2. The control group progressed to a peak titer at 35 days after the start of therapy, then fell thereafter. The group on 0.89 mg./Kg./day had a slower rise in titer, but it was still increasing at 80 days after therapy was started. All other groups showed early rises and falls of titers. Results with the tpcf 50 test are shown in figure 3. The control group and the one on 0.89 mg./Kg./day rose to high mean titers early and remained there. All other groups rose to only low titers, where they stayed.

The general condition of most rabbits remained good with no significant losses of weight. There have been deaths in the groups at 3.6, 7.1, 14.3, 28.6, and 114 mg./Kg./day. However, no consistent findings occurred in their physical conditions antemortem or postmortem.

FIG. 2. Effect of dextrosulphenidol in early syphilis of rabbits: VDRL slide test.

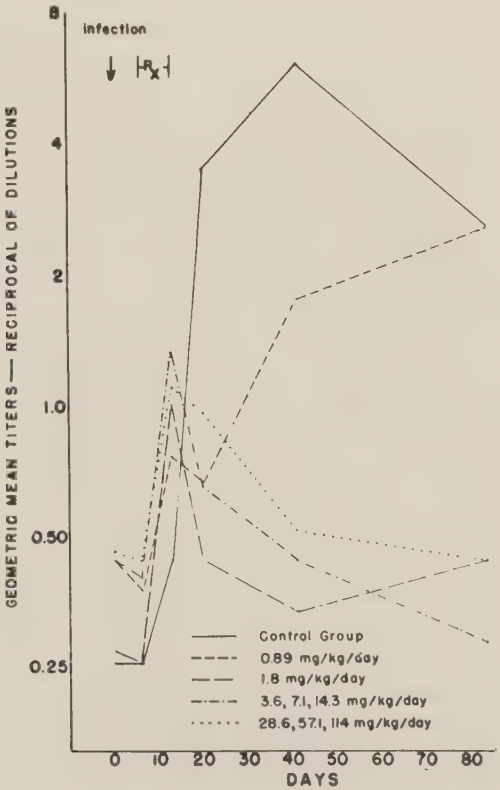




FIG. 3. Effect of dextrosulphenidol in early syphilis of rabbits: tpcf 50 test.

DISCUSSION

The results obtained to date indicate that dextrosulphenidol apparently cured early syphilis of rabbits over a range of dosages from 3.6 to 114 mg./Kg./day. This decision was based on early healing of cutaneous lesions and lack of secondary syphilomas in these groups. Cure was also based on early falls of titers in the VDRL slide test and low titers in the tpcf 50 test. Definitive conclusions in regard to biological cure of syphilis must await the results of the transfers of popliteal lymph nodes at six months after infection.

Two aspects of the data deserve special remark. The first is the rather short time for the attainment of dark-field negativity in the animals on higher dosages of dextrosulphenidol. The mean period in these groups was three days, and 2 rabbits achieved a negative status at 36 hours. A similar study has been performed in this laboratory with chloramphenicol. Table I shows the comparison of chloramphenicol with dextrosulphenidol in the production of dark-field negativity. The rabbits that received a dosage of chloramphenicol of 114 mg./Kg./day attained dark-field negativity at only three days after the start of treatment. However, lower dosages of this drug produced slower clearing of treponemes from the lesions than did the same doses of dextrosulphenidol.

Another significant comparison is the minimal curative dosage for early experimental syphilis. Using dextrosulphenidol, it is apparently 3.6 mg./Kg./day. Similar data for chloramphenicol (table II) show the minimal curative dosage to be about

114 mg./Kg./day, which confirms previous observations of Kolmer.⁵ This 32-fold difference in curative dosage is significant when considering toxic manifestations of drugs to be used in clinical trials. For example, in comparison with the total dosage of 12 to 24 Gm. of chloramphenicol required to cure human syphilis, it could conceivably take as little as 1 to 2 Gm. of dextrosulphenidol.

SUMMARY

1. This is a preliminary report on the first use of dextrosulphenidol in experimental syphilis.

2. Intramuscular administration of the drug was made every 12 hours for seven days at dosages from 0.89 to 114 mg./Kg./day. Evaluations of the drug's efficacy were performed by periodic clinical observations and serological tests.

3. The data give tentative indication that the minimal curative dose of dextrosulphenidol for experimental syphilis is 3.6 mg./Kg./day.

Confirmatory evidence of biological cure at this level of drug is awaited with interest for comparison with chloramphenicol. In this study dextrosulphenidol had no greater evidence of toxicity than chloramphenicol.

4. Should confirmation of these findings emerge in further studies, cautious clinical evaluation of dextrosulphenidol in human syphilis would appear to be indicated.

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A Report on the Use of Kanamycin in Experimental Syphilis

THOMAS C. WASHBURN, WARFIELD GARSON, AND JOHN W. CLARK, JR.

Venereal Disease Experimental Laboratory, Venereal Disease Branch, Communicable Disease Center, Public Health Service, and School of Public Health, University of North Carolina, Chapel Hill, N. C.

The most effective antibiotic therapy for syphilis is still penicillin. However, the increasing use of penicillin in many infectious diseases has led to more persons becoming hypersensitive to penicillin. The serious consequences of reactions to penicillin have been well discussed by Guthe et al.¹ Other antibiotics have been used to treat syphilis, as reviewed by Olansky and Garson,² but none has shown penicillin's efficacy. Intramuscular forms of new antibiotics are being investigated for potential use in clinic patients.^{3,4} Their lack of reliability in taking oral forms of drugs has been discussed by Olansky et al.⁵

This paper reports on a study of the effects of intramuscular kanamycin* in experimental syphilis of rabbits. Kanamycin is a recently developed antibiotic whose characteristics have been extensively investigated.⁶ This study of kanamycin was prompted by its structural similarity to streptomycin, which has shown antitreponemal activity in high dosages.⁷

EXPERIMENTAL METHODS

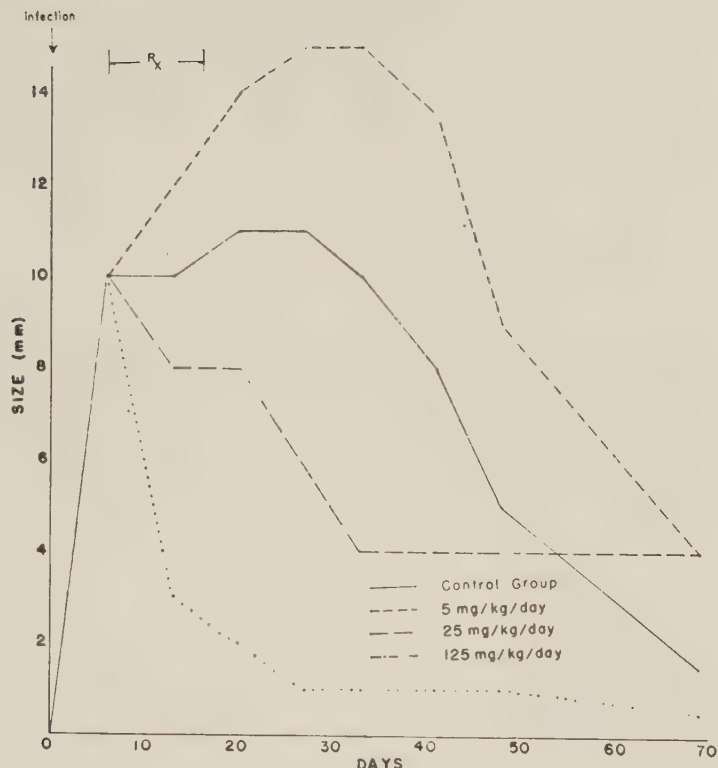
In this study, methods were derived from work previously described by Garson and McLeod³ and Turner and Schaeffer.⁸ Young adult male rabbits of mixed breeds were inoculated intracutaneously at eight sites on their shaven backs with a suspension of *Treponema pallidum* (Nichols strain). The organisms were derived from the testes of rabbits that had been inoculated intratesticularly 7 to 10 days previously. The organisms were extracted into a mixture of equal parts of normal rabbit serum and physiological saline. The gross tissue particles were removed by centrifugation at 500 G. for 10 minutes. The supernatant fluid contained the suspension of *T. pallidum*. There were about 7.5×10^7 organisms/ml. of suspension, as determined on a calibrated dark-field microscope. Each site on the rabbits' backs received 0.2 ml. of this suspension.

At six days after infection, most rabbits had dark-field-positive cutaneous lesions that measured 5 to 10 mm. in diameter. Kanamycin was started on the sixth day and administered intramuscularly every eight hours for 10 days. The drug was given to three groups of 6 rabbits each at dosages of 5, 25, and 125 mg./Kg./day (total dosages of 50, 250, and 1250 mg./Kg.). The other infected group (control) received no drug.

Kanamycin's therapeutic effect was evaluated clinically and serologically. Clinically, the evolution of the cutaneous lesions and the occurrence of secondary

* The trade name of Bristol Laboratories for kanamycin is Kantrex. The drug was supplied for this study by this firm.

FIG. 1. Effect of kanamycin in early syphilis of rabbits: Mean diameters of cutaneous lesions.



syphilomas in skin or testes were closely observed. The cutaneous lesions were described and measured daily for three weeks, then periodically for another six weeks. The testes were periodically palpated to determine the presence of indurated or swollen masses during the first three months of infection. At least one cutaneous lesion/rabbit was incised to obtain fluid for dark-field examination on the days when lesions were observed. Fluid for dark-field examination was aspirated from the indurated or swollen testicular masses. Rabbits were considered to have dark-field-negative lesions if they were negative on three consecutive days. Rabbits were said to be in clinical relapse if dark-field-positive secondary lesions occurred.

Serological evaluation of kanamycin's effect was made by observing the time course of titers of two tests, VDRL slide test⁹ and tpcf 50 test.^{10*} Serum for tests was obtained before infection and just prior to treatment, then periodically for 10 more weeks.

Lymph node transfers were performed at six months after infection. This was done with the control group and the clinically negative rabbits. Both popliteal lymph nodes were emulsified in 50 per cent normal rabbit serum in saline, and the mixture was transferred to one testis each of 2 normal rabbits. Possible infection in the recipients of the lymph nodes was evaluated by periodic palpation of the testes. Fluid was aspirated from suspicious masses for dark-field examination. The serological tests were performed on the recipients' blood taken before the transfers and at three months after the transfers. All testes that were still negative at three

* Tests were performed by Dr. J. Portnoy's section.

TABLE I

*Effect of Kanamycin in Early Syphilis of Rabbits:
Time for Lesions to Convert to Dark-field Negativity*

Animal group	Days from start of drug therapy
Control	48
5 mg./Kg./day	47
25 mg./Kg./day	29
125 mg./Kg./day	5

months were emulsified in saline, and this suspension was subjected to dark-field examination. Negative findings in these tests were considered to indicate "cure" of syphilis in the original donors of lymph nodes.

Evaluation of possible toxicity of kanamycin for rabbits was made by observing the weights, the equilibrium, and the sites of injection.

RESULTS

The evolution in size of the cutaneous lesions is shown in figure 1. Although the lesions of the control group remained large for 20 days, they had regressed to a nearly healed state by 60 days after the start of therapy. The group at 125 mg./Kg./day had healed lesions (mean diameter of 1 mm.) by 20 days with no evidence of relapse. The group at 25 mg./Kg./day had healing lesions earlier than the control group. Then relapses began to occur at about 20 days after treatment was started, and the size of lesions remained at a mean diameter of 4 mm. The group at 5 mg./Kg./day had large lesions that regressed later than those of the control group.

The dark-field data are presented in tables I and II. The number of days from the start of therapy to the first of three consecutive negative tests is shown in table I. The groups at 125 and 25 mg./Kg./day had shorter times to negativity than did the control group, but the group at 5 mg./Kg./day had the same time. Table II shows the number of days after start of therapy to the occurrence of dark-field-positive secondary lesions. Only the group at 125 mg./Kg./day did not have secondary syphilomas.

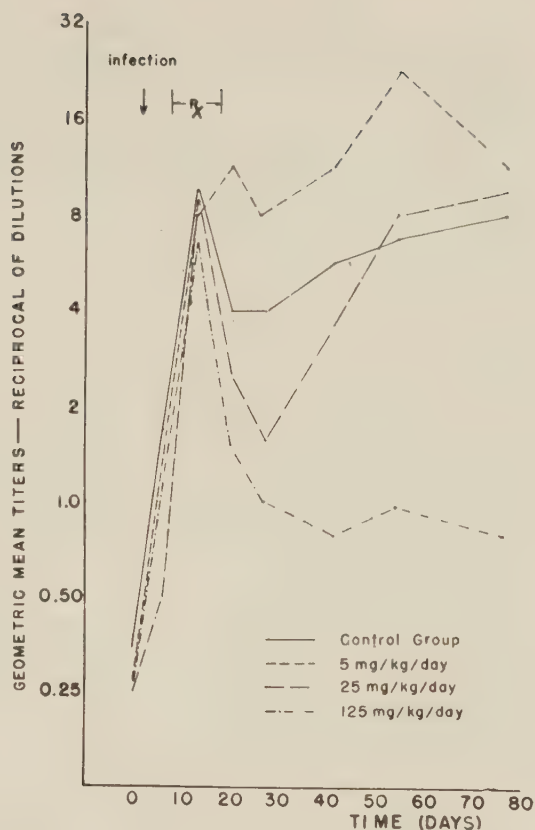
The results of the serological tests are presented in figures 2 and 3. In the VDRL slide tests, shown in figure 2, two treated groups had falls in titer while the control group remained at a high level. The titers of the group at 125 mg./Kg./day con-

TABLE II

*Effect of Kanamycin in Early Syphilis of Rabbits:
Evidence of Secondary Lesions*

Animal group	Days from start of drug therapy
Control	29
5 mg./Kg./day	27
25 mg./Kg./day	28
125 mg./Kg./day	No secondary lesions

FIG. 2. Effect of kanamycin in early syphilis of rabbits: VDRL slide test.



tinued to fall to a mean titer of about 1. The groups at the lower levels of drug evidenced rises in titer by 35 days and reached a titer near that of the control at 70 days after therapy was started. The tpcf 50 tests are shown in figure 3. The control group and the group at 5 mg./Kg./day rose to high titers and stayed there. The group at 25 mg./Kg./day rose more slowly to about the same titer as the control group. The group at 125 mg./Kg./day rose to a much lower titer and stayed there.

The transfers of lymph nodes from all control rabbits produced syphilis in the recipients. The transfers from the group at 125 mg./Kg./day produced no syphilis in the recipients.

No systemic or local toxic effects of kanamycin were detected. Temporary losses of weight occurred in all groups, but most rabbits recovered their pretreatment weights. At least one death occurred in each group, but more rabbits died in the group at 25 mg./Kg./day than in the others.

DISCUSSION

The results show rather clear-cut differences between the group at 125 mg./Kg./day and the control group. The lesions of this therapeutic group healed far more rapidly and reached a dark-field-negative state 40 days before those of the controls. The group at 125 mg./Kg./day had no secondary lesions and no positive

transfers of lymph nodes. The serological tests show faster regression of titers and lower titers in the group at 125 mg./Kg./day than in the controls. In essence, the rabbits that received a dosage of kanamycin of 125 mg./Kg./day were cured of syphilis.

Different results were attained with the other two therapeutic groups. The group at 25 mg./Kg./day had earlier regression of lesions toward healing than the control group. However, dark-field-positive secondary relapses occurred in all rabbits of this therapeutic group. The primary lesions of this group reached dark-field negativity at 20 days, but dark-field-positive secondary lesions had already occurred by then. The serological titers tended to be less in this therapeutic group than in the controls for about 35 days after the drug was started. After that time, the titers of the two groups were nearly the same. In other words, kanamycin at a level of 25 mg./Kg./day produced early but unsustained healing of syphilis. The group at 5 mg./Kg./day showed somewhat larger lesions, later healing of lesions, and higher serological titers than the control group. Other studies in this laboratory have led to the belief that these latter observations did not indicate a significant difference between the two groups.

Toxicity of kanamycin for rabbits was evidenced only by temporary losses of weight. Deaths that occurred were apparently not related to the administration of kanamycin at these dosages. However, it would not be practicable to use kanamycin at 125 mg./Kg./day in human beings because of potential toxicity to the kidneys and auditory nerves.⁶

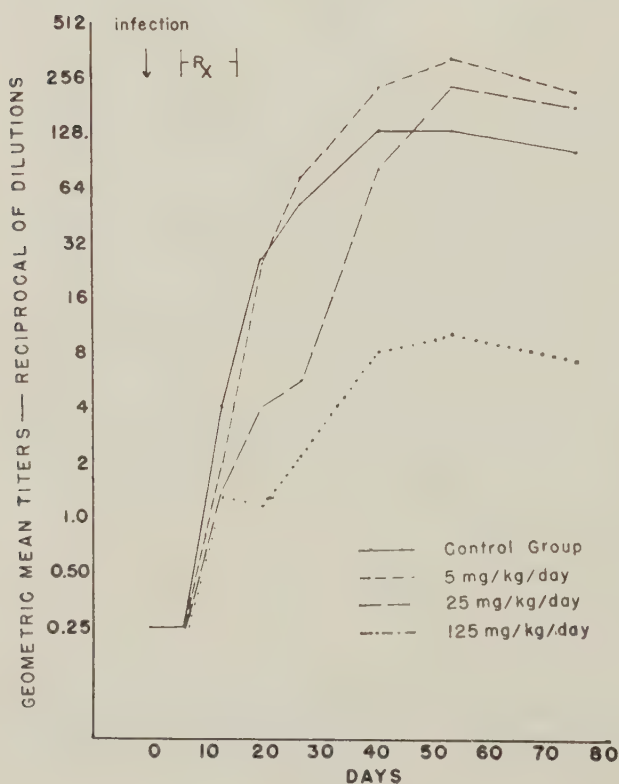


FIG. 3. Effect of kanamycin in early syphilis of rabbits: tpcf 50 test.

SUMMARY

Kanamycin has been used for the first time in the treatment of early syphilis in rabbits. Cure from syphilis has been evaluated by clinical and serological tests and by transfers of lymph nodes. The results showed that kanamycin given at 125 mg./Kg./day for 10 days cured the rabbits. Similar schedules of 25 and 5 mg./Kg./day produced no cures. Should a comparable dosage in human beings be necessary for cure of syphilis, the potential toxicity of this agent would preclude its use in most instances.

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Treatment of Contagious Syphilis with a Tetracycline-Oleandomycin Combination

FLORENCIO PRATS AND MARCO A. DE LA PARRA

Department of Dermatology, University of Chile, and Department of Dermatology, Jose Joaquín Aguirre Clinical Hospital, Santiago, Chile

Following a plan of examining all new antibiotics for their antisyphilitic action, we tested a tetracycline-oleandomycin combination* therapeutically in 31 cases of contagious syphilis, 4 of which were cases of mixed infection (syphilis plus lymphogranuloma inguinale).

The principal object of this study was to find an antibiotic that is effective against syphilis and that could take the place of other antisyphilitic medications, such as penicillin, since serious phenomena of hypersensitivity to the latter antibiotic are being observed with an ever-increasing frequency.

Another aim of this clinical and therapeutic evaluation of the tetracycline-oleandomycin combination was to find, if possible, an antisyphilitic drug that would be harmless, principally in that no serious Herxheimer reaction would occur with its use. It is well known that such reactions occur during penicillin therapy. We also were looking for an antibiotic that would be effective against mixed infections such as syphilis and lymphogranuloma inguinale.

MATERIAL AND METHOD

Thirty-one cases of contagious syphilis were treated, 9 of which were primary and 22 secondary. Lymphogranuloma inguinale was a concomitant condition in 4 of the 31 cases, viz., in 3 cases of primary and 1 case of secondary syphilis.

Three of the patients were pregnant women; 1 of them was one and one half months gravid and had primary syphilis, the other 2 (four and eight months gravid, respectively) had secondary syphilis (table I).

The patients with primary and secondary syphilis received the tetracycline-oleandomycin combination orally, in capsules of 250 mg. each, the dosage being 2 capsules (500 mg.) every six hours for 10 days.

In the mixed cases (syphilis plus lymphogranuloma inguinale), administration of the drug was continued for an additional 10 days, using a dosage of 1 Gm. daily (1 capsule of 250 mg. every six hours.)

The action of the drug was studied by means of the examinations that are ordinarily carried out for the assay of antisyphilitic medicaments, viz., negative dark field examination, healing of the lesions, and serological regression.

RESULTS

Negative Dark Field Examination. This was accomplished in 26 patients. The time at which the findings became negative is indicated in figure 1. This was

* The trade name of Chas. Pfizer & Co. for the 2:1 mixture of tetracycline-oleandomycin is Signemycin.

TABLE I

Case Material

Patient no.	Sex	Type of syphilis	Concomitant condition	Dark field examination	Kahn test, units
1	F	Primary		+	20
2	M	Primary		+	3
3	M	Primary		+	2
4	M	Primary		+	10
5	F	Primary			2
6	F	Primary	Pregnancy, 1½ months	+	20
7	M	Primary	Lymphogranuloma inguinale	+	10
8	M	Primary	Lymphogranuloma inguinale	+	10
9	M	Primary	Lymphogranuloma inguinale	+	20
10	F	Secondary			2
11	F	Secondary		+	2
12	M	Secondary		+	1
13	M	Secondary		+	40
14	M	Secondary		+	3
15	M	Secondary		+	16
16	M	Secondary		+	20
17	F	Secondary			40
18	M	Secondary		+	20
19	M	Secondary		+	32
20	F	Secondary		+	20
21	F	Secondary		+	30
22	M	Secondary			2
23	F	Secondary		+	10
24	M	Secondary		+	256
25	F	Secondary		+	10
26	F	Secondary		+	60
27	F	Secondary		+	4
28	M	Secondary		+	40
29	F	Secondary	Pregnancy, 4 months	+	16
30	F	Secondary	Pregnancy, 8 months	+	100
31	M	Secondary	Lymphogranuloma inguinale	+	50

studied in 26 of the 31 patients. It could not be done in 5 patients for various reasons. It was established that the findings turned negative in most cases (16 patients) in less than 39 hours, the minimum and maximum periods being 8 and 49 hours, respectively.

Healing of the Lesions. In cases of primary syphilis, cicatrization of the lesions was observed about the fifteenth day. In 8 cases of secondary syphilis, cicatrization

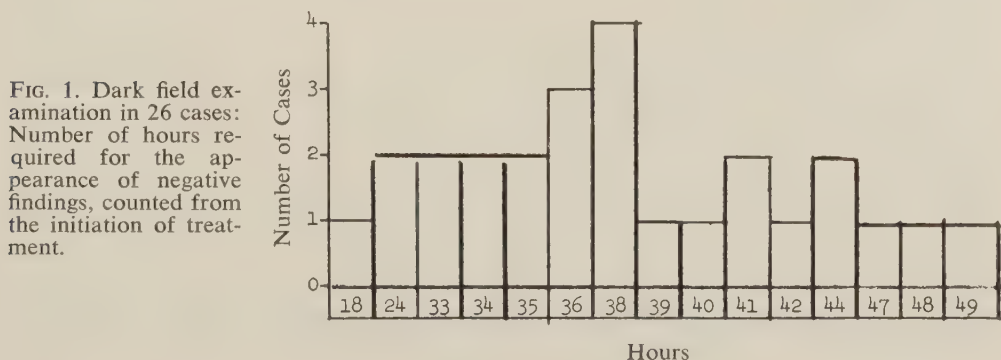


TABLE II

Seventeen Cases in Which It Was Possible to Check on Serological Regression

Patient no.	Kahn test, units	Month																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	10																		0		
2	3	4	1																		
3	32			0																	
4	2												0								
5	10																	0			
6	1									0											
7	40							0													
8	3							3													
9	50				0																
10	16				0																
11	2				0																
12	2		±																		
13	10		0																		
14	100	32																			
15	60	16																			
16	2									0											
17	16				0																

of the lesions was complete, or they were almost fully healed, between the tenth and twelfth days.

In the mixed cases (syphilis plus lymphogranuloma inguinale), cicatrization was delayed in comparison with the other cases; the average length of the delay varied from five to seven days.

Serological Regression. Serological study was performed in 17 patients. In 12 of them, complete negativity was obtained (Kahn). Two of the others received only one month of treatment, and the regression was manifested in a decline from 100 to 32 Kahn units and from 60 to 16 units, respectively. The findings in the other cases were 1, 8, and 3 units, respectively, and these patients have received two, four, and seven months of treatment, respectively (table II).

TOLERANCE

The drug was very well tolerated in all cases. Two patients who had been sensitive to penicillin, with very violent anaphylactic reactions, were placed on the tetracycline-oleandomycin combination and did not develop any sensitivity reaction to this therapy.

When penicillin is replaced by another antibiotic, a study of this phenomenon is therefore of the utmost interest. We had previous experience in this respect with erythromycin, and it was important to know what the results would be with the tetracycline-oleandomycin combination in this case.

Herxheimer Reaction. When penicillin is administered, the frequency of Herxheimer reactions is approximately 90 per cent in contagious cases, less in latent syphilis. These reactions are often intense enough to produce high temperatures and severe general manifestations.

The Herxheimer reaction was observed in 15 patients who received the tetracycline-oleandomycin combination, but the symptoms observed were merely mod-

TABLE III

Fifteen Cases in Which the Herxheimer Reaction Appeared

Patient no.	Temperature	Other complaints
1		Headache
2	38.5	Ostealgia
3	37	
4	38.2	
5	37	
6	37.2	
7	37.1	Headache and gastralgia
8	38.5	
9	37.3	
10	37.2	
11	37	Headache
12	37	
13	37.5	
14	37.4	Headache
15	37	

erate—a slight rise in temperature and, in a few cases, other complaints of minor degree (table III).

SYPHILIS AND PREGNANCY

Emphasis should be made of the excellent results obtained in the treatment of pregnant patients with the tetracycline-oleandomycin combination.

Case 1. The patient was one and one half months' pregnant and had primary syphilis, acquired 30 days previously. The dark field examination became negative in 38 hours. The serology, which was 20 Kahn units at the initiation of treatment, has not been rechecked since.

Case 2. The patient was eight and one half months' pregnant and had secondary syphilis, first onset. Serology was 100 units. Despite the advanced state of the pregnancy and the probable severe syphilis of the fetus, the same dosage of the tetracycline-oleandomycin treatment was used in order to provide comparative data and also to observe clinically how effective this drug could be.

The dark field examination became negative in 39 hours. Delivery took place three days after the termination of tetracycline-oleandomycin therapy. The infant was premature, weighing 2.400 Kg. and measuring 44 cm. in length, but appeared healthy and showed no syphilitic lesions. The physical examination was negative, except for a moderate hypertrophy of the liver and a percussible and palpable spleen.

Naturally, owing to the short period of time that elapsed between the end of therapeutic cure and the birth, the child probably is not completely normal in every respect. He is being kept under observation at the present time, and so far has appeared clinically healthy and normal. In the mother, the serology was 32 units 40 days after the initiation of the treatment. She is still under observation.

Case 3. In a case of recrudescant secondary syphilis, the ultramicroscopy was positive initially. Serology was 16 units. The patient was five months' pregnant. The findings of the dark field examination became negative in 33 hours.

Delivery took place four months after the termination of treatment. The infant appeared to be normal and weighed 2.800 Kg. 20 days after birth. He has undergone a check-up including roentgenograms at the National Health Service since then, and his condition is good at this time.

Four combination cases were treated, 3 of them had primary syphilis; the fourth, secondary syphilis. All patients began treatment with positive serology; the findings with regard to the syphilitic infection have become negative in 3 patients, whereas the fourth has not been re-examined since then.

As for the lymphogranulomatous infections in these patients, we were able to observe total regression (disappearance of lesions) in an average of 25 days.

SUMMARY AND CONCLUSIONS

Thirty-one patients with contagious syphilis, including 4 with mixed infection (syphilis and lymphogranuloma inguinale) and 3 pregnant patients, were treated with the tetracycline-oleandomycin combination, given orally in capsules, using a dosage of 2 Gm. daily for a period of 10 days. The treatment was continued for 10 more days, using a dosage of 1 Gm. daily, in the mixed infections.

The condition of the patients was observed clinically as well as by laboratory study, the therapeutic evaluation being based on the following criteria: the negativity of findings of the dark field examination, healing of the lesions, and serological regression evidenced by the Kahn test (the latter could be performed only in 17 cases).

Cicatrization of the lesions was observed in all patients. It occurred about the fifteenth day in the cases of primary syphilis and between the tenth and twelfth days in patients with secondary syphilis, whereas it was delayed five to seven days more in the mixed infections. Dark field examinations were negative in all patients in less than 49 hours; the serology, studied in 17 patients, became completely negative in 12, while it showed a distinct decrease in the other 5, which were the most recent cases.

Cure of the syphilis was achieved in the 3 pregnant patients. Two of them have already given birth. One of these 2, who first received treatment in the fifth month of pregnancy, had a normal child, whereas the other, treated at the end of her pregnancy, has a child who looks "premature" but who has no evident syphilitic lesions.

In the cases of mixed infections, both infections were cured; the average time required for the disappearance of the manifestations of lymphogranuloma was 25 days.

Manifestations attributable to the Herxheimer reaction appeared in only 15 of the 31 patients. Generally, such manifestations consisted of a light or moderate fever or other very mild symptoms.

This antibiotic combination was very well tolerated by all patients and did not give rise to any toxic manifestation; in an occasional patient side effects of only minor degree appeared.

In view of the excellent curative action of the tetracycline-oleandomycin combination in contagious syphilis, whether primary or secondary, as well as in the mixed infections in which the patients also had lymphogranuloma inguinale, and considering, moreover, the excellent tolerance of this combination by oral administration, with only rare, mild manifestations of the Herxheimer reaction as compared with other antibiotics, we are of the opinion that this preparation constitutes an active and safe medication for the treatment of contagious syphilis.

Absorption of Orally Administered Neomycin and Kanamycin, with Special Reference to Patients with Severe Hepatic and Renal Diseases

CALVIN M. KUNIN,* THOMAS G. CHALMERS, CARROLL M. LEEVY,† SUSAN C. SEBASTYEN, CHARLES S. LIEBER, AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital, the Medical Services, Lemuel Shattuck Hospital, and the Departments of Medicine, Harvard Medical School and Tufts University School of Medicine, Boston, Mass.

Oral preparations of neomycin and kanamycin are extensively used for intestinal antiseptics because of the wide spectrum of their activity against many intestinal microorganisms, and their limited absorption from the gastrointestinal tract. These drugs are frequently used in patients with severe liver disease for the treatment or prophylaxis of hepatic coma. Of some importance is the known fact that patients in liver failure are usually oliguric and thus potentially more susceptible to nephrotoxic agents. If such patients either absorb more, or retain more after absorbing normal amounts, they may be more likely to develop severe renal failure.

The present study was designed to provide additional information concerning the absorption of neomycin and kanamycin from the gastrointestinal tract in man, particularly in patients with severe hepatic or renal disease in order to define the circumstances in which unusually high concentrations may be encountered.

The concentrations of neomycin in the serums of 6 normal subjects who had no demonstrable hepatic or renal impairment given a single oral dose of 4 Gm. of the sulfate were determined. With the exception of 1 patient, a small but variable amount of drug was absorbed. The peak levels were noted at one to four hours, low levels were still present at eight hours, but none were demonstrable at 24 hours after the oral dose. The mean peak concentration was 4 $\mu\text{g./ml.}$

The mean urinary recoveries of these drugs in 24 hour collections were: for normal subjects, 0.6 and 0.7 per cent of the administered dose of neomycin and kanamycin respectively; for patients with cirrhosis of the liver, 0.5 and 1.1 per cent of the administered dose of the same drugs respectively.

The concentrations of neomycin and kanamycin in the blood of patients with cirrhosis of the liver who received 8 Gm./day of either drug for the treatment or prophylaxis of hepatic coma were correlated with the nonprotein nitrogen level in blood obtained on the same day. Antibiotic concentrations in 22 samples from patients with normal nonprotein nitrogen values ranged from indeterminate to 9.5 $\mu\text{g./ml.}$, whereas in 22 samples from patients whose nonprotein nitrogen was greater than 50 mg./100 ml., antibiotic was detectable in the serum of all those studied, and in 7 of them the levels were in excess of 10 $\mu\text{g./ml.}$, the upper limit of normal in this series. Highest serum levels (up to 44 $\mu\text{g./ml.}$) were noted with neomycin,

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The full text and data of this paper will appear soon in the *New England Journal of Medicine*.

* Present address: University of Virginia Medical College, Charlottesville, Va.

† Present address: Seton Hall College of Medicine, Jersey City Medical Center, Jersey City, N.J.

but fewer uremic patients receiving kanamycin were studied. In 2 uremic patients receiving neomycin followed serially, a gradual increase in serum levels was noted.

A patient with severe renal disease without superimposed cirrhosis of the liver was given 6 Gm. of neomycin per day for three days. During this period, drug was detectable in his serum and continued to rise despite cessation of therapy so that on the sixth day his serum level was 10 μ g. ml. Thus, in the presence of impaired renal excretion neomycin given by the oral route tends to accumulate in the blood.

It would appear from these observations that caution must be exercised in administering oral neomycin or kanamycin to patients with renal failure. Both drugs have been shown to be nephrotoxic,^{1,2} although kanamycin may be less so. It may be preferable to use the latter drug in clinical situations that appear to require good intestinal antisepsis when the patient is uremic.

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Studies on the Streptomycin and Dihydrostreptomycin Methioninates

I. Antibacterial Action on Sensitive and Resistant Strains of *Mycobacterium tuberculosis*

FERNANDO DAS NEVES ALMEIDA AND JOSÉ MANUEL DAS NEVES ALMEIDA

Instituto de Assistência Nacional aos Tuberculosos, Lisbon, Portugal

The work of Carvalho and Ribeiro¹ seems to demonstrate that the reduced toxicity of streptomycin methioninates, previously described by Alves et al.,² is not due to a lowering in activity, i.e., does not result from inactivation of streptomycin by methionine. It was important to prove this because streptomycin action may be inhibited by some thiol derivatives. Accordingly it appeared necessary to study the action of the 20 per cent mixture of streptomycin and dihydrostreptomycin methioninates with streptomycin and dihydrostreptomycin sulfates* on a sufficient number of strains of *Mycobacterium tuberculosis*. The studies of Carvalho and Ribeiro¹ were made with *Bacillus subtilis* and the results might be different with *Myco. tuberculosis*. Since there may be great variability even within the same bacterial species, we worked with 24 strains.

MATERIALS AND METHODS

Strains. H₃₇Rv and 23 other strains of *Myco. tuberculosis* were used. Most of them had recently been isolated from patients. The strains were subcultured on Lowenstein Jansen medium, ground with glass pearls, and nephelometrically standardized.

Medium. Tarshis medium (25 per cent blood agar) was employed. The antibiotic solutions were mixed in the solid medium at 50 C.

Antibiotic Concentrations. The solutions were prepared in order to obtain the following concentrations of streptomycin per ml. of medium: 100, 50, 20, 10, 5, 2, 1, and 0 µg. The test series contained the 20 per cent mixture of streptomycin and dihydrostreptomycin methioninates with streptomycin and dihydrostreptomycin sulfates; the control series contained the mixture of streptomycin and dihydrostreptomycin sulfates.

Technique of Determination. We used our own technique,³ briefly as follows: using the antibiotics included in the medium, we soaked discs of filter paper (6 mm. in diameter) in the inoculum and then put them on the medium. In this way, we could test several strains with one series of media (in this work we cultured eight strains on each Petri dish). After having inoculated all the discs, we hermetically

* The trade name of Atral Laboratories, Lisbon, for this mixture is Streptionin. Composition is 10 per cent streptomycin methioninate, 10 per cent dihydrostreptomycin methioninate, 40 per cent streptomycin sulfate, and 40 per cent dihydrostreptomycin sulfate.

TABLE I

Sensitivity to Streptomycin Sulfate of the Strains Tested

Number of strains	Sensitivity, $\mu\text{g./ml.}$
6*	1
3	2
2	5
2	10
2	20
5	50
4	100

*In this group is included strain H₃₇Rv.

sealed the dishes with adhesive tape. The reading was made after three weeks, when the growth could be seen around and over the discs.

RESULTS

In table I are shown the degrees of resistance to streptomycin of the strains studied.

The results obtained with the 20 per cent mixture of methioninates of streptomycin and dihydrostreptomycin were similar to those with streptomycin. There were differences of only one dilution—sometimes in favor of the methioninates, sometimes the sulfates—but such differences have no significance, as is well known.

SUMMARY

The writers have found that bacteriostatic concentrations in 24 strains of *Myc. tuberculosis* of the 20 per cent mixture of streptomycin and dihydrostreptomycin methioninates and streptomycin and dihydrostreptomycin sulfates are similar to those of the sulfates alone.

The trials were made with Tarshis medium (25 per cent human blood agar). The strains used covered all grades of streptomycin resistance.

These findings prove that the decreased toxicity of the methioninate mixture tested is not related to a reduction in antibacterial action.

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Studies on the Streptomycin and Dihydrostreptomycin Methioninates

II. Tolerance by Patients with Intolerance to Streptomycin Sulfates

FERNANDO DAS NEVES ALMEIDA AND JOSÉ MANUEL DAS NEVES ALMEIDA

Instituto de Assistência Nacional aos Tuberculosos, Lisbon, Portugal

Streptomycin and dihydrostreptomycin methioninates were synthesized by Alves et al¹ in 1958. These drugs have a lower toxicity than streptomycin and dihydrostreptomycin sulfates. Accordingly, we decided to use them in patients with signs of toxicity caused by the injection of streptomycin sulfate and dihydrostreptomycin sulfate.

MATERIALS AND METHODS

Patients. Tuberculous patients admitted to the sanatorium and having previously presented toxic symptoms due to streptomycin were used in the study. All patients stopped treatment with streptomycin until the disappearance of symptoms. Treatment was resumed with sulfate without the patient knowing which drug was administered. There was a reappearance of the toxicity symptoms in all patients. The symptoms were dizziness, headache, and facial paresthesias. There was a total of 44 patients.

Drugs. We have used three different concentrations of methioninates: a mixture of equal parts of streptomycin and dihydrostreptomycin methioninates, a 50 per cent mixture of the methioninates and streptomycin and dihydrostreptomycin sulfates, and a 20 per cent mixture of the methioninates and streptomycin and dihydrostreptomycin sulfates. Simultaneously *p*-aminosalicylic acid, isoniazid, or both were administered, as recommended for the treatment of tuberculosis.

Dosage. All drugs tested were injected in a dosage equivalent to 1 Gm. of streptomycin daily.

Follow-up of Patients. The patients, who did not know which drug was injected, were carefully observed at least once a week. They were not asked about toxicity symptoms, but if the patients referred to them, the symptoms were clinically observed daily. If we ascertained that the complaints were due to the treatment, it was stopped.

RESULTS

Until now the patients have been injected with different dosages averaging 152 Gm. Only 6 of 44 patients had to stop treatment. The tolerance of the treatment plotted against time is shown in figure 1. For comparison the figure also shows the curve for 20 per cent streptomycin pantothenates (based on figures taken from Neves Almeida and Neves Almeida²).

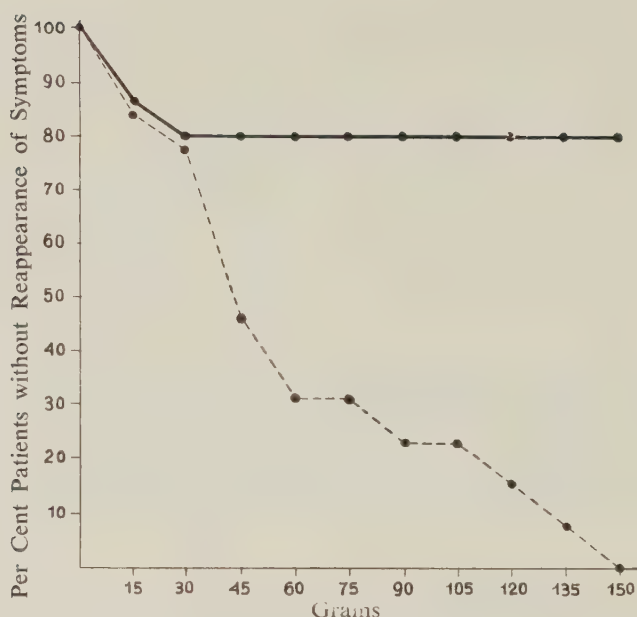


FIG. 1. Percentage of patients without reappearance of symptoms of intolerance, medicated with the 20 per cent pantothenate mixture (—) and the 20 per cent methioninate mixture (----).

DISCUSSION

The 20 per cent mixture of streptomycin and dihydrostreptomycin methioninates with streptomycin and dihydrostreptomycin sulfates is usually well tolerated by patients showing toxic symptoms due to streptomycin. Only 6 of 44 (14 per cent) patients had to stop the treatment. The action of the mixture in lowering toxicity seems superior to that of the pantothenates of streptomycin and dihydrostreptomycin in equal concentration. This is easily seen in figure 1.

The mechanism by which methionine lowers the toxicity of streptomycin remains unknown, but it seems to be different from that of the pantothenates. Actually, as can be seen in figure 1, the emergence of toxicity with the two drugs is very dissimilar both in length of time and in frequency.

Tolerance to the methioninates of streptomycin by patients with intolerance to the sulfates will permit continuation of streptomycin therapy after the appearance of toxic symptoms. At present, with the tendency for daily use of streptomycin in conjunction with isoniazid, intolerance to streptomycin by the tuberculous patient will be more and more frequent and early in appearance. Because of this the methioninates of streptomycin are particularly important.

SUMMARY

The writers studied the following products: a mixture in equal parts of streptomycin and dihydrostreptomycin methioninates, a mixture of 50 per cent methioninates and streptomycin and dihydrostreptomycin sulfates, and a mixture of 20 per cent methioninates and streptomycin and dihydrostreptomycin sulfates. The methioninates and the 50 per cent mixture are poorly tolerated, provoking continuous pain at the site of injection.

The experiments were continued using only the 20 per cent mixture. This was well tolerated when injected daily in a dosage equivalent to 1 Gm. of streptomycin by 38 of 44 patients who had previously shown intolerance to streptomycin. A total dosage averaging about 150 Gm. has been given so far.

When injected with streptomycin sulfate, all these patients had shown one or more of the following symptoms: dizziness, headache, and facial paresthesias, disappearing when the treatment was stopped and reappearing when the drug was taken again.

In equal concentrations streptomycin methioninate appears superior to streptomycin pantothenate and deserves a place in the treatment of tuberculous patients with intolerance to streptomycin.

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Studies on the Streptomycin and Dihydrostreptomycin Methioninates

III. Tolerance by Patients Submitted to Long-Term Daily Therapy—Preliminary Report

FERNANDO DAS NEVES ALMEIDA AND JOSÉ MANUEL DAS NEVES ALMEIDA

Instituto de Assistência Nacional aos Tuberculosos, Lisbon, Portugal

The most powerful treatment of tuberculosis seems to be the combination of isoniazid and streptomycin, administered daily. The greatest drawback of this regimen is the toxicity of streptomycin. Indeed, this toxicity depends on total dosage and not on length of treatment, and thus it appears sooner if the drug is injected daily. Consequently it is important to determine whether the methioninates of streptomycin and dihydrostreptomycin^{1,3} increase the tolerance in long-term treatment.

We have been treating a group of 55 tuberculous patients, who had never received streptomycin or had received a maximum of 20 Gm. of the antibiotic, with a 20 per cent mixture of streptomycin and dihydrostreptomycin methioninates and streptomycin and dihydrostreptomycin sulfates.* These patients have also been taking isoniazid daily (4 to 5 mg./Kg. of body weight). The 20 per cent mixture of the methioninates of streptomycin has been injected daily in a dose equivalent to 1 Gm. of streptomycin.

The great majority of patients have now had 120 days or more of treatment, and in some cases the injected dose has reached 150 Gm.

Up to this writing, the tolerance has been excellent by all patients. The clinical results have been good and in all cases comparable to results to be expected of the combination therapy used.

Although acknowledging that the period of treatment has been very short, the writers believe it has been sufficient for the appearance of several cases of toxic symptoms if the tolerance of the drug studied were not better than that of streptomycin and dihydrostreptomycin sulfates.

The study is now being continued.

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* The trade name of Atral Laboratories, Lisbon, for this combination is Streptionin. Composition is: 10 per cent streptomycin methioninate, 10 per cent dihydrostreptomycin methioninate, 40 per cent streptomycin sulfate, and 40 per cent dihydrostreptomycin sulfate.

The Comparative Toxicity and Clinical Effectiveness of Vancomycin, Ristocetin, and Kanamycin

BURTON A. WAISBREN, LEONARD KLEINERMAN, JOSEPH SKEMP,
AND GLENN BRATCHER

Infectious Disease Control Unit, Department of Medicine, Milwaukee County General Hospital, and the Marquette University School of Medicine, Milwaukee, Wis.

Patients with severe staphylococcal infections that have not responded to the usual antimicrobial therapy are commonly seen in present-day hospital practice. It is hoped that the data to be presented regarding the relative efficacy and toxicity of ristocetin, vancomycin, and kanamycin will help the clinician make a decision about further antimicrobial therapy for these patients. It was possible to alternate the use of ristocetin, vancomycin, and kanamycin in a group of seriously ill patients because the three drugs were discovered within such a short time of each other that no one of them had an opportunity to be established as "the drug of choice" in severe staphylococcic infections.¹⁻³ Each of these three antibiotics acted as a control for the others in the study, and this circumvented the necessity of extrapolating opinions regarding their effectiveness in severe infections from actual experience with milder, non-life-threatening infections.

MATERIALS AND METHODS

Drugs. Ristocetin was discovered in 1956 by a group of workers at Abbott Laboratories in Chicago under the direction of W. E. Grundy.¹ All clinically isolated staphylococci apparently are sensitive to ristocetin's action and it has had particular use in staphylococcal pneumonia and enterococcal bacterial endocarditis.^{4,5} It can be administered only by the intravenous route, and best results are said to be obtained if approximately 1 Gm., dissolved in approximately 150 ml. of 5 per cent dextrose and water, is given by rapid intravenous injection. In this study some pilot plant material was used, but most of the patients were treated with commercially available ristocetin.

Vancomycin was discovered in 1956 by a group of workers at the Lilly Laboratories in Indianapolis, Ind., under the direction of M. H. McCormick.² All staphylococci from clinical isolates are sensitive to vancomycin's action and it has been used in a wide variety of staphylococcal infections with excellent results.^{2,6,7} Vancomycin is administered intravenously in daily dosages of 2 Gm./day dissolved in 500 to 1500 ml. of 5 per cent dextrose and water. Both pilot plant and commercially available vancomycin were used in this study.

Kanamycin was discovered by Umezawa and co-workers in Japan and its commercial development was by Bristol Laboratories of Syracuse, N. Y.³ All clinically isolated staphylococci are sensitive to kanamycin's action; data regarding its clinical effectiveness were presented at a symposium of the New York Academy of Medicine in September, 1958.⁸

Comparative studies regarding the sensitivity to the three drugs of 75 strains of pathogenic staphylococci revealed that in vitro kanamycin was more active than

TABLE I

Age, Sex, Race, and Place of Onset of 3 Groups of Patients Treated with Vancomycin, Kanamycin, or Ristocetin

Antibiotic	Sex		Mean age, yr.	Race		Place of onset	
	Male	Female		White	Negro	Home	Hospital
Kanamycin	27	23	54	41	9	31	19
Vancomycin	31	23	54	51	3	31	23
Ristocetin	29	22	50	44	7	33	18

were ristocetin and vancomycin, although all strains were sensitive to 6 µg./ml. of all three drugs. Cross resistance studies also have shown that vancomycin, ristocetin, and novobiocin are closely related but that there is relatively little relationship between kanamycin, vanomycin, and ristocetin.⁹

Selection of Patients. Patients were given either vancomycin, ristocetin, or kanamycin for one of the following reasons: Staphylococci isolated from their wounds were resistant in vitro to other antibiotics;¹⁰ there was no response to other antibiotics; the infection was so severe that therapy had to be started before sensitivity studies would be available; or the patients were hypersensitive to other antibiotics. Whenever one of the drugs was used, the clinical status of the patient justified, in our opinion, the use of potentially toxic drugs that had not been fully investigated. Strict alternation was not accomplished for several reasons. The most important was that the investigators were influenced by results in individual patients and found themselves unable to maintain complete objectivity in certain cases. For instance, after renal deaths due to kanamycin and vancomycin, a tendency to shy away from these drugs in patients with renal disease developed. Similarly, after complete agranulocytosis developed from ristocetin, its use was not resumed for several weeks. However, when at this time equally severe toxic reactions were noted with the other antibiotics, the alternation with ristocetin was resumed. Ristocetin was used in most cases of endocarditis because of its published success in this disease and our early failures with the other drugs.⁵ Undoubtedly, stricter alternation could have been practiced in a series of patients with furuncles or mild wound infections, but it was felt that the lack of strict alternation was the price that had to be paid to do a comparative study using seriously ill patients. On the other hand,

TABLE II

Previous Infections Suffered by Patients Who Received Vancomycin, Ristocetin, and Kanamycin

Previous infection	No. of kanamycin-treated patients	No. of vancomycin-treated patients	No. of ristocetin-treated patients
None	35	35	30
Pneumonia	4	8	4
Urinary tract	5	3	5
Dental	2	2	1
Skin	3	4	3
Bronchitis	0	0	5
Central nervous system	2	0	1
Appendicitis	1	1	0

TABLE III

Secondary Diagnoses of Patients Treated with Ristocetin, Vancomycin, and Kanamycin

Secondary diagnosis	Kanamycin, no. of patients	Vancomycin, no. of patients	Ristocetin, no. of patients
Neoplasm	6	8	9
Postoperative state	8	15	7
Heart disease	12	9	15
Gastrointestinal disorder	1	4	3
Vascular disease	8	5	9
Diabetes	4	1	4
Fractures and dislocations	10	10	10
Central nervous system disease	10	8	5
Renal disease	6	4	6
Liver disease	2	3	3
Alcoholic disease	4	5	1
Decubitus ulcers	34	6	2
Traumatic wounds (gun and knife)	2	0	0
Burns	0	2	0
Malnutrition	3	1	2
Pregnancy	0	3	1
Gout	0	0	1
Anemia	1	0	0
Poisoning (arsenic)	0	0	1
Myelofibrosis	0	1	0
Tuberculosis	6	1	1
Glaucoma	1	0	0

similarities in the ages, sex, races, and other pretreatment data suggest that random sampling of a sort, at least, was obtained.

A total of 131 patients were treated. Fifty-four patients were treated with vancomycin, 51 with ristocetin, and 50 with kanamycin. The total number of patients was only 131 because some patients were treated with either two or three of the drugs. Ninety-six patients were seen on the wards of the Milwaukee County General Hospital and 35 were seen in consultation and treated in one of seven private hospitals in Milwaukee County. All patients, except 5 with endocarditis, were suffering from bacteriologically proved staphylococcal infections.* Sixty of the 131 patients died, and this is a measure of the severe therapeutic test that was given. Forty-six of these 60 patients died from the staphylococcal infection and 14 died primarily from underlying diseases, such as cancer, leukemia, and heart failure.

The groups of patients treated with each antibiotic were compared in regard to age, sex, race, previous therapy, primary and secondary diagnosis, and the results of therapy. In vitro sensitivity studies of the majority of the strains isolated from the patients were done by the tube dilution method.¹¹ All patients were personally observed during the course of their illness by one of the authors, and all hospital charts were carefully scrutinized.

RESULTS

Two types of results will be presented. The first is an analysis of the patients studied in regard to how well the method of case selection used was able to provide comparative groups of patients and second are the actual results of the administration of the drugs to each group of patients.

* Coagulase-positive *Staphylococcus aureus*.

TABLE IV

Primary Diagnoses of Patients Treated with Ristocetin, Kanamycin, and Vancomycin

Primary diagnosis	Ristocetin-treated group, total*/survivors	Kanamycin-treated group, total/survivors	Vancomycin-treated group, total/survivors
Pneumonia	20/7	12/6	24/14
Endocarditis	13/10	4/1	5/2
Bacteremia	11/3	13/6	8/5
Pyelonephritis	3/1	11/5	1/0
Enteritis	2/1	2/2	8/6
Osteomyelitis	7/6	3/3	1/1
Abdominal abscess	3/1	5/4	3/2
Central nervous system infection	3/1	1/0	1/1
Wound infection	6/3	7/6	5/4
Septic pericarditis		1/1	
	68/33	59/34	56/35

* The totals are slightly higher than the numbers of patients treated with each antibiotic because some of the patients were treated for more than one of the major diagnoses.

Comparisons Between the Groups of Patients Treated with Ristocetin, Vancomycin, and Kanamycin. The mean age of the 51 patients treated with ristocetin was 50 years, the mean age of the 54 patients treated with vancomycin was 54, and the mean age of the 49 patients treated with kanamycin was 54 (table I). There were 9 Negroes treated with kanamycin, 3 with vancomycin, and 7 with ristocetin (table I). There were a few more men than women treated with each drug. The percentages of hospital and home infections were similar in each treatment group. Analysis of the previous infections suffered by the patients treated with each of

TABLE V

Comparison of Laboratory Data Determined Before, While, and After

	Hemoglobin		Hematocrit		White blood count		% polymorpho-nuclear leukocytes		% eosinophils	
	No.	Means	No.	Means	No.	Means	No.	Means	No.	Means
<i>Before Treatment</i>										
Ristocetin	119	10.52	90	36.33	108	13.6M	104	76.21	75	2.11
Vancomycin	99	11.4	69	38.61	89	12.8M	80	74.29	69	1.20
Kanamycin	135	10.69	95	36.35	123	12.2M	117	75.12	90	2.14
<i>During Treatment</i>										
Ristocetin	93	10.77	73	36.99	109	13.1M	102	73.33	83	2.37
Vancomycin	77	10.96	57	36.61	74	13.2M	69	73.96	49	2.65
Kanamycin	67	10.68	44	36.32	67	15.3M	62	73.26	58	2.55
<i>After Treatment</i>										
Ristocetin	113	10.28	62	37.10	125	11.1M	108	62.19	90	3.38
Vancomycin	68	11.44	50	35.70	69	12.9M	65	69.55	49	1.96
Kanamycin	80	10.13	46	37.12	87	12.4M	78	63.50	75	3.69

the antibiotics showed patient resistance prior to serious staphylococcal infection to be remarkably similar in each group (table II). Table III shows the secondary diagnoses of the patients in each group and tends to show also that the patients treated with each drug had similar complicating problems.

The primary diagnoses in the three groups of patients are shown on table IV. The bias in case selection is evident from this table. Ristocetin was used in 13 cases of endocarditis, whereas only 5 patients were treated with vancomycin, and 4 with kanamycin. More patients with pneumonia were treated with vancomycin and ristocetin than with kanamycin, whereas more urinary tract infections were treated with kanamycin. This bias has already been discussed (*vide supra*) and it is felt to be a significant but unavoidable accompaniment of a therapeutic study of patients who were as sick as those treated (table IV).

Comparison of the mean values of laboratory data determined before, during, and after therapy is given in table V. All available determinations were used. It is apparent that on the basis of all laboratory studies, the three groups of patients were similar (table V).

The previous antibiotic therapy received by the patients in each group is seen on table VI. With few exceptions the agents had been used in full therapeutic dosage and had failed to give a therapeutic response before ristocetin, kanamycin, or vancomycin was used. The fact that the majority of patients had received at least two antistaphylococcal drugs before being treated with ristocetin, kanamycin, or vancomycin was another measure of the severe test given to each of these agents.

The duration of therapy and dosage used in each group of patients are shown

TABLE V
Patients Were Given Ristocetin, Vancomycin, or Kanamycin

Nonprotein nitrogen		Chlorides		Carbon dioxide		Sodium		Potassium		Total protein A/G	
No.	Means	No.	Means	No.	Means	No.	Means	No.	Means	No.	Means
86	57.39	61	100.3	60	22.83	57	138.33	54	4.14	27	6.50 3.15/ 3.35
76	38.34	54	99.63	50	24.06	51	136.43	49	4.18	21	6.73 3.36/ 3.37
86	37.49	55	97.48	53	25.37	54	136.59	52	3.95	22	6.79 3.28/ 3.56
67	40.80	37	101.31	32	21.70	34	136.21	33	4.17	8	6.40 3.29/ 3.11
79	38.03	39	99.14	38	23.71	40	135.01	36	4.03	10	6.23 2.75/ 2.48
72	40.43	32	97.79	32	23.33	30	137.12	31	4.09	8	6.27 2.96/ 3.31
78	44.13	35	99.66	35	22.31	34	134.42	35	4.39	9	6.75 3.44/ 3.31
49	50.67	27	100.11	20	22.80	25	136.80	25	4.22	8	7.1 3.18/ 3.92
55	47.22	19	99.21	19	22.73	19	134.49	19	3.83	7	6.83 3.15/ 3.68

TABLE VI

*Antibiotics Received by Patients Before Either Ristocetin, Kanamycin,
or Vancomycin Was Administered*

Antibiotic	Ristocetin group, no. of patients	Vancomycin group, no. of patients	Kanamycin group, no. of patients
Penicillin	35	36	32
Erythromycin	23	27	23
Chloramphenicol	33	41	38
Tetracyclines	15	6	13
Nitrofurantoin*	4	5	9
Neomycin	15	19	10
Streptomycin	19	4	11
Novobiocin	5	5	3

*Not an antibiotic.

on table VII, and these data show that a slightly higher dosage of ristocetin was used than with the other drugs but this may be compensated for by the fact that it was not used for quite as long a time. Thus, all in all, the total amounts of drugs used in each group of patients were very similar (table VII).

When tables I through VII are scrutinized, they suggest that even with the limitations of the methods of case selection already discussed, the groups of patients treated with the three drugs were remarkably similar in regard to age, race, sex, severity of infection, pretreatment laboratory determinations, and previous therapy. In addition, the patients received comparative amounts of antibiotics on a weight-for-weight basis.

Comparative Results of Therapy. The over-all conclusion in regard to the results of therapy was based on the considered opinion of the physician who had administered the drug and on the objective evidence of response yielded by a careful study of the patient's chart. Disappearance of fever, negative cultures, improvement in laboratory studies, and beneficial changes in the patient's clinical condition all were considered. In about one fifth of the cases a decision as to whether the drug actually helped the patient could not be made.

Ristocetin was effective in 17 of 51 cases, vancomycin in 21 of 54, and kanamycin in 17 of 49 (table VIII). Definite therapeutic failure was noted in 23 of 51 cases with ristocetin, 22 of 54 with vancomycin, and 23 of 49 with kanamycin (table VIII). These figures certainly do not suggest that any one of the three drugs is any more effective than the others when they are considered on an over-all basis (table VIII).

When no over-all differences were noted, other specific factors of response were

TABLE VII

*Duration of Therapy and Dosages Used in Patients Given Ristocetin,
Vancomycin, and Kanamycin*

	Mean duration of therapy, days	Mean dosage, Gm.	Mean daily dosage, Gm.
Ristocetin	7.3	16	2.2
Vancomycin	10.7	18	1.68
Kanamycin	9.6	16	1.64

TABLE VIII

Clinical Response Noted with Ristocetin, Vancomycin, and Kanamycin

	Total patients treated	Number of clinical responses	Number of clinical failures	Undetermined
Ristocetin	51	17 (33%)	23	11
Vancomycin	54	21 (39%)	22	11
Kanamycin	50	17 (37%)	23	9

compared. These included comparisons between the mean times between onset of therapy and the first afebrile day, the number of positive cultures obtained from various sites during and after therapy, mean temperatures during therapy, laboratory determinations during therapy, and over-all toxic reactions. Twenty-six patients receiving ristocetin became afebrile in a mean time of 6.9 days. Thirty-one patients receiving vancomycin became afebrile in a mean time of eight days, and 30 patients receiving kanamycin became afebrile in the mean time of 9.6 days. Only survivors were used in these calculations. We do not feel these figures to be suitable for statistical analysis, but it is possible that if the slightly longer time it took for response to kanamycin is significant, it is due to the fact that kanamycin is administered intramuscularly, whereas ristocetin and vancomycin are given by vein. The mean time between treatment and control of the infection, as judged by the entire clinical picture, was 16.5 days with ristocetin, 16 days with vancomycin, and 17.6 days with kanamycin.

Comparison between numbers of cultures positive for coagulase-positive staphylococci is seen in table IX. It should be noted that blood cultures were positive during and after therapy with each of the drugs. This does not mitigate their effectiveness since we have seen the same thing happen with penicillin therapy against penicillin-sensitive staphylococci, but others who have noted this in a few patients treated with ristocetin have reported bacteremia as more characteristic with this

TABLE IX

Comparisons Between Total Numbers of Cultures Positive for Staphylococci Before, During, and After Therapy with Ristocetin, Kanamycin, and Vancomycin

Source	Before	During	After
Vancomycin			
Blood	15	3	3
Stool	7	4	0
Sputum	11	17	3
Other	43	21	27
Total	76	45	33
Ristocetin			
Blood	15	3	3
Stool	5	4	2
Sputum	10	14	9
Other	50	22	19
Total	80	43	33
Kanamycin			
Blood	12	1	2
Stool	7	2	0
Sputum	7	9	3
Other	52	11	22
Total	78	23	27

TABLE X

Efficacy of Ristocetin, Vancomycin, and Kanamycin After Failure with One of These Antibiotics

	Number of times effective after failure with:			Number of times not effective after failure with:		
	Ristocetin	Vancomycin	Kanamycin	Ristocetin	Vancomycin	Kanamycin
Ristocetin	—	—	1	—	1	3
Vancomycin	1	—	4	3	—	—
Kanamycin	—	—	—	4	3	—

drug.¹¹ Our data do not support this contention. There were fewer positive cultures while patients were receiving kanamycin, but a simple Chi square test shows the difference not to be significant and after therapy the number of positive cultures was similar for all three drugs.

In 17 cases, one of the other antibiotics under study was administered when either ristocetin, vancomycin, or kanamycin had failed to achieve a response (table X). In 6 of these 17 cases a response was obtained, five times with vancomycin and once with ristocetin. Two of the 4 cases that responded to vancomycin after failure with kanamycin were of septicemia. The others were a severe cellulitis at the site of a skin graft and a case of staphylococcal enteritis. These data are not sufficient to support the concept of vancomycin as a superior drug, but they do indicate that it may be worth while to try another antistaphylococcal antibiotic when the one being used is not effective, even when, as in these cases, the causative bacteria are sensitive to both agents *in vitro*.

Other comparisons of objective evidence regarding response and toxicity are seen in tables V and XI. One positive finding appears to be the lower urine outputs with kanamycin in the limited number of patients for whom these figures were available. This, in our opinion, does not represent toxicity but rather indicates that fluids were given to the patients for the intravenous administration of ristocetin and vancomycin, whereas the kanamycin was given by the intramuscular route. Thus, analysis of the laboratory data did not reveal any over-all significant differences among the groups of patients receiving ristocetin, kanamycin, and vancomycin. However, the mean blood nonprotein nitrogen concentrations did rise after therapy with kanamycin and vancomycin (table V).

All strains of staphylococci isolated from this series of patients were sensitive to

TABLE XI

Objective Comparisons of Data Obtained During and After Patients Received Ristocetin, Vancomycin, and Kanamycin

	Temperature mean during	Albuminuria of 2+ or more, no. of cases			Urine output per day, ml.	
		Before	During	After	During	After
Ristocetin	99.82	19	8	8	1194 (7)*	1268
Vancomycin	100.17	12	6	4	1594 (7)	1537
Kanamycin	99.9	20	14	8	808 (9)	893

* Numbers in parenthesis show number of cases averaged.

TABLE XII

Evidences of Toxicity with Ristocetin, Vancomycin, and Kanamycin

Reaction	Ristocetin	Vancomycin	Kanamycin
None	37	46	45
Generalized rash	8	2	1
Exfoliation of skin	3	0	0
Deafness	1	1	1
Severe renal damage	1 (1)*	3 (1)*	3 (3)*
Agranulocytosis	1	0	0
Shaking chills and shocklike picture	0	2	0
Neutropenia	2	0	0
Total	16	8	5

* Fatal.

6 µg./ml. or less of the three antibiotics. However, to determine whether there was a better response when the bacteria were extremely sensitive, the results of the sensitivity tests were plotted against the clinical results. This study showed that organisms isolated from treatment failures were not even relatively more resistant to the antibiotics than those isolated from patients in whom a clinical response was achieved and indicated that antibiotic resistance apparently was not the cause of treatment failure.

Toxic reactions with each drug are summarized in table XII and it is apparent that toxic reactions were a problem with all three drugs. Five deaths were directly attributable to drug toxicity; the 131 patients treated suffered a total of 29 instances of toxicity (22 per cent). The magnitude of this figure can best be understood when one considers that all these patients had received other antibiotics. The only toxicity from these were the development of a staphylococcic diarrhea in 10 of the patients, most of whom had received tetracycline, and the development of 1 case of deafness from neomycin, which was used in the patient with agranulocytosis. The toxicity with each drug will be detailed and brief representative case summaries presented.

RISTOCETIN. Thirty-seven of 51 patients treated with ristocetin had no toxic reactions. Sixteen toxic reactions were encountered in the other 14 patients. The most common reaction was a generalized measles type of rash that appeared in 8 patients shortly after the onset of treatment. The rash looked exactly like that seen after novobiocin therapy and faded in several days when the drug was discontinued.¹³ Three* of the patients in this series suffered complete exfoliation of the skin.

CASE 1. The patient was a 40 year old woman with subacute bacterial endocarditis that had not responded to massive dosages of penicillin, streptomycin, and chloramphenicol. She was started on ristocetin, 1 Gm. intravenously in 500 ml. of 5 per cent dextrose and water twice daily. After the second dose she developed the characteristic generalized rash, but due to her septic condition and the failure of the other antibiotics, the ristocetin was continued. The rash increased daily in intensity in spite of 50 mg. of tripelethamine hydrochloride given orally before each infusion. After seven days of treatment (14 Gm.), the skin began to exfoliate and at this time all antibiotics were discontinued. Within the ensuing two weeks the patient lost all the skin on her body. However, there was no weeping or fluid loss associated

* We have seen another case of complete exfoliation in a patient treated successfully for acute endocarditis.

with the exfoliation. The new skin was soft and pliable; lanolin was used liberally to protect her during this time. No further treatment was given and she went on to uneventful recovery. A leukemoid reaction appeared simultaneously with the rash and the white blood count rose from 11,100 to 36,400 with 91 per cent polymorphonuclear leukocytes. Never were more than 3 per cent eosinophils found. Blood urea nitrogen concentration and hemoglobin remained within normal limits.

CASE 2. The patient was a 42 year old white woman with bilateral pulmonary fibrosis who was given ristocetin for acute staphylococcal pneumonia. Nine Gm. of ristocetin was given over a five day period, and this was discontinued and erythromycin started by mouth when the organism proved sensitive to the latter drug. A generalized rash was noted on the last day of the therapy with ristocetin, and this became confluent and was followed by a non-weeping exfoliation of all the patient's skin. She was treated conservatively with lanolin, and soft pliable skin was noted under the exfoliation. Response to the ristocetin was excellent and the course of the pneumonia uneventful. The eosinophil percentage of white blood cells never went above 4 per cent and platelet counts were normal during and after the administration of the ristocetin.

CASE 3. The patient was a 48 year old Negro man with acute staphylococcal endocarditis. He was given a daily dosage of ristocetin of 2 Gm. On the sixth day of this program he

TABLE XIII
Summary of Laboratory Studies of Case 3

Date	Risto- cetin, daily dosage, Gm.	Risto- cetin blood level*	Non- protein nitrogen	Hemo- globin	White blood count	Poly- morpho- nuclear leuko- cytes	Lymph- ocytes	Eosino- phils	Platelets
March 26	2		22.5	9	5300	73	24		
March 27	2	20							
March 28	2	41							
March 29	2								
March 30	2								
April 1	2								
April 2	3								
April 3	3	164							
April 4	3	328	53	7	6250	56	29	4	
April 5	3								
April 6	3								
April 7	3								
April 8	3			8	1800	0	83	15	
April 9				7	1200	0	68	20	210,000
April 10				6.7	1500	0	73	15	
April 11					1750				
April 12					2350				
April 13				7	3200				
April 17					5000	0	96		596,000
April 18					4450				
April 19					2850				
April 22					1550	1	84	3	
April 23					5000	1	89	6	328,000
April 24					1400				
April 25					1000	1	84	2	
April 26					1000	0	87	1	
April 27					2250		90	6	
April 29					1500	4	82	8	
April 30					1800	5	46		
May 1				7.6	3000	17	43	2	
May 5				6	5400	72	13		284,000

* These blood levels were determined by Dr. Sylvestor of Abbott Laboratories.

developed a generalized skin rash. It was decided to try to continue therapy in spite of the rash, which evolved in the next three weeks to a complete exfoliative dermatitis, which did not weep, and which responded to local lanolin administration and therapy with diphenhydramine hydrochloride. On the thirteenth day of therapy the patient developed a complete agranulocytosis. He was isolated and maintained on kanamycin, and when after 18 days no polymorphonuclear cells had appeared in his blood, he was started on 60 mg. of Metacortone per day. Forty-eight hours after the first administration of Metacortone, polymorphonuclear leukocytes were again seen, and 72 hours later, 17 per cent of the white blood cells were polymorphonuclear leukocytes. At the end of eight days the count was 75 per cent polymorphonuclear leukocytes. The patient went on to complete recovery although he did become completely deaf after neomycin therapy, which was started toward the end of the period of agranulocytosis. Complete studies of the mechanism of this agranulocytosis are being published elsewhere.¹⁴ These studies suggest a possible abnormality in the sulfhydryl metabolism of this patient's leukocytes. It is of interest that the platelet count remained normal during the entire episode. The high ristocetin blood levels also are worthy of note, as is the rise of the nonprotein nitrogen that occurred during therapy. This might be interpreted to mean that a high level was reached due to renal insufficiency, which caused a toxic reaction rather than an idiosyncrasy. On the other hand, the high eosinophil count favors an idiosyncrasy or it may have been due to the exfoliative dermatitis. (Table XIII.)

Three patients who received ristocetin developed depression of the granulocytic series. One of these developed agranulocytosis but the other 2 developed transient falls in the polymorphonuclear count, which returned to normal when the drug was stopped. The patient with agranulocytosis (case 3) also suffered exfoliation.

CASE 4. The patient was a 55 year old white woman who was given ristocetin, 2 Gm. daily, for an acute staphylococcal endocarditis and a large splenic abscess. She made a clinical response, but during the four days of therapy the white blood count dropped from 32,400 to 5000 white blood cells/cu. mm. The percentage of polymorphonuclear cells was 78 per cent initially and 73 per cent when the blood count had dropped. Therapy with ristocetin was discontinued, and one week later the count had risen again to 7900 and three weeks later it was 25,000. It is possible that the fall was due to a therapeutic response, but more likely that it was due to the drug. The case is detailed here to emphasize that if daily white blood counts are taken, the fall in count can be stopped early and therapy discontinued. This will apparently prevent a complete agranulocytosis. The patient had no rise in the blood nonprotein nitrogen concentration during this time.

One patient became deaf within 24 hours after the administration of ristocetin.

CASE 5. The patient was a 72 year old white man with chronic osteomyelitis of the spine and staphylococcal bacteremia (this complicated case has been reported in detail elsewhere¹⁵). The patient had tolerated previous courses of neomycin, chloramphenicol, and vancomycin without undue toxicity. He was one of the first patients to receive ristocetin, and after a single dose of 1 Gm. of ristocetin, he began to complain of difficulties in hearing. He was given a 10 day course of 1 Gm./day and by its end was completely deaf. He has never regained his hearing. During this 10 day course the patient was anemic but renal function appeared normal. He had received a two week course of vancomycin two weeks before the start of the ristocetin and no loss of hearing had been noted after this therapy.

One patient had a recurrence of a renal shutdown when it became necessary to treat a severe staphylococcal pneumonia with ristocetin.

CASE 6. The patient was a 56 year old white man who was admitted to the hospital with a renal shutdown due to arsenic ingestion. He was treated conservatively, and by the twenty-second hospital day the nonprotein nitrogen concentration had fallen from a high of 171 to 42 mg./100 ml. and urine output was 950 ml./day. The patient was started on ristocetin, 1.5 Gm./day in 1000 ml. of 5 per cent dextrose in water, when a bronchopneumonia did not

TABLE XIV
Summary of Laboratory Data of Case 6

Date	Dosage of ristocetin, Gm.	Nonprotein nitrogen	Urine output
Feb. 11	1.5	46	980
Feb. 12	1.5	40	1000
Feb. 13	1	43	1500
Feb. 14	1	14	700
Feb. 15	1	68	10
Feb. 16			250
Feb. 17			200
Feb. 18		121	>200
Feb. 19	Died		

respond to erythromycin and chloramphenicol. He received a total of 6 Gm. of ristocetin when he again stopped producing urine, and the nonprotein nitrogen rose. At autopsy both toxic nephrosis and bilateral bronchopneumonia were demonstrated. The renal failure might not have been due entirely to the ristocetin because the patient was just recovering from tubular damage due to arsenic and was suffering from staphylococcal pneumonia. However, the fact remains that he received ristocetin, stopped producing urine, and died in an oliguric state. The platelet count remained normal. (Table XIV.)

Our experience with toxicity from ristocetin can be summarized by stating that skin toxicity was most commonly observed. This responded to conservative measures. In addition, deafness, agranulocytosis, leukopenia, and renal failure were all observed in the relatively small groups of patients treated. Thrombocytopenia, as reported by Gangarosa et al,¹⁶ was not seen although repeated platelet counts were done on most of the patients. Phlebitis was not recorded, but over-all it was not a serious problem, particularly when the drug was dissolved in 300 ml. of 5 per cent dextrose in water and infused rapidly.

VANCOMYCIN. Forty-six of the 54 patients treated with vancomycin suffered no toxic reactions. Two patients had a generalized rash similar to that seen with ristocetin, but no exfoliation occurred. Two patients had a severe chill and shocklike reaction. Three patients developed severe nitrogen retention, and 1 developed a transient deafness. There was 1 death, due apparently to renal failure from the vancomycin.

CASE 7. The patient was a 40 year old white woman with serum hepatitis who had been receiving tetracycline, 1 Gm./day, for one week when she developed diarrhea and increase in fever. A Gram stain of the stool showed many gram-positive cocci, and she was given 1 Gm. of vancomycin in 500 ml. of 10 per cent glucose solution. She tolerated this well. Eighteen hours later she was given 1 Gm. of vancomycin in 1000 ml. of 10 per cent glucose. When 500 ml. of this infusion had been given, she developed a severe chill, shortness of breath, and a temperature of 105 F. (oral). The infusion was discontinued. The temperature became normal in three hours, the diarrhea stopped, the stools then showed normal flora, and she went on to uneventful recovery. There was no rise in blood nonprotein nitrogen level.

We have seen this type of sudden chill and shocklike reaction on four other occasions and apparently it was associated with several lots of vancomycin. Vials with the same lot numbers were tested in animals and no pyrogens or unusual characteristics were found.¹⁷

Three cases of renal toxicity all occurred in relatively young patients who presumably had normal kidneys before the onset of the illness. All were extremely ill.

CASE 8. The patient was a 45 year old white woman with an acute staphylococcal pneumonia of the entire left lower lobe of the lung who was given vancomycin, 1 Gm. twice a day, dissolved in 300 ml. of 5 per cent dextrose in water. She made an excellent clinical response but within five days she developed twitching and incoherence and the blood urea nitrogen rose from 7 to 34 mg./100 ml. All parenteral medication was stopped and she was given neomycin by aerosol.¹⁸ The blood urea nitrogen remained elevated during her entire hospital stay but four weeks later it was 14 mg./100 ml. Urine and other blood studies did not seem to be affected during this time, and the initial symptoms that we attributed to acute nitrogen retention passed away within 72 hours after the vancomycin was stopped. It is of interest that the phenolsulfonphthalein test was within normal limits, even while she still had nitrogen retention. The pneumonia cleared without incident. (Table XV.)

CASE 9. The patient was a 38 year old woman who, when first seen on May 18, 1959, appeared moribund from an acute posthysterectomy staphylococcal peritonitis. She was given, by infusion, 3 Gm. of vancomycin in 300 ml. of 5 per cent dextrose in water, 4 Gm. of chloramphenicol succinate in 300 ml. of 5 per cent dextrose in water, 20 ml. of gamma globulin intramuscularly, and 6 ml. of lanatoside intravenously. The vancomycin was given from 6:00 p.m. to 8:00 p.m. and at 7:00 p.m. she developed a hard, shaking chill associated with tachycardia (a rate of 150 to 170 beats/minute) and dyspnea. The hourly urine output from 7:15 to 8:15 was 60 ml., but from 8:15 to 9:15 it was only 30 ml. and after that was less than 5 ml./hour until about 11 o'clock the next morning, when the urine output slowly started to return. She remained oliguric for the next 48 hours when the urine output again improved. In spite of this, the blood nonprotein nitrogen concentration rose to a height of 81 mg./100 ml. and then gradually fell. It was still significantly elevated when the patient was discharged. She was given no more vancomycin after the initial dose but was maintained on chloramphenicol and erythromycin. Eighteen hours after the beginning of the vancomycin therapy the patient seemed markedly improved clinically in spite of her lack of urine output. The temperature was 106 F. (rectal) when the vancomycin was started. It rose to 107 F. (rectal) five hours later and was only 103 F. the next morning. At this time the patient complained of difficulty in hearing. This was progressive over the next 48 hours but then her hearing improved concomitant with the twice daily injection of pyridoxine (50 mg.), which was continued to the end of her hospital stay. At that time her hearing was normal. The toxic nephrosis, paralytic ileus, and subdiaphragmatic and pelvic abscesses were then treated by the accepted techniques and the patient was discharged from the hospital 19 days after she received the vancomycin.

TABLE XV

Summary of Laboratory Data on Case 8

Date	Dosage of vancomycin, Gm.	Blood urea nitrogen, mg./100 ml.	Creatinine, mg./100 ml.	Phenolsulfonphthalein
May 14	2	7		
May 15	2			
May 16	2	34	3.6	
May 17	2			
May 18	2	37		
May 19		30	4.1	
May 20		36	3.8	
May 21		39	3.9	
May 22		34	3.5	
May 23		28	3.2	
May 25		27	2.5	
May 26		33	2.5	
May 27		38	2.5	
May 29		38	3.2	
June 1		25	1.8	
June 23		14		

29.4%
2 hr. total

TABLE XVI
Summary of Laboratory Data on Case 9

Hourly urine output for 13 hours after 3 Gm. of vancomycin													
Time, hours	1	2	3	4	5	6	7	8	9	10	11	12	13
Ml. of urine	60	70	30	15	<5	<5	<5	<5	<5	<5	<5	<5	<5

Date	Nonprotein nitrogen	Output
May 18	36	300
May 19	51	371
May 20		400
May 21		1000
May 22		2000
May 23		2200
May 24	81	2400
May 25		2500
May 26		2300
May 27		3000
May 28	61	2500
May 29		4000
May 30		2000
June 3	61	
June 8	59	

Therapy in this case was extreme due to the patient's condition, but the case does seem to represent severe toxic nephrosis and deafness most likely due to the vancomycin. The technique of recording hourly urine output allowed a rapid diagnosis and prevented us from giving additional vancomycin, which might have been fatal. (Table XVI.)

CASE 10. The patient was a 41 year old white man with subacute bacterial endocarditis who was given vancomycin, 2 Gm./day, for 13 days. He developed progressive nitrogen retention during this time although all other signs of activity of the endocarditis improved. The vancomycin was stopped when nitrogen retention became apparent but the patient continued on to oliguria and died from what was considered to be both renal and heart failure. It could be argued that the renal status was due to the endocarditis, but it is reasonable to suppose that the vancomycin also had a part in the fatal outcome. Unfortunately, permission for autopsy was refused. This patient suffered severe chills and fever after the first two infusions of vancomycin but tolerated subsequent infusions without overt evidence of toxicity. (Table XVII.)

The experience with the toxicity of vancomycin can be summarized by stating that renal damage was the most common manifestation of toxicity and in 1 case it was felt to be a contributing cause in the death of the patient. A transient rise in the blood nonprotein nitrogen concentration was noted frequently in patients who received vancomycin, and this is mirrored by the data in table V, which show the mean nonprotein nitrogen concentrations to have risen from 38 to 50 mg./100 ml. after therapy. This was not considered a major evidence of toxicity because the rise was transient and did not appear to harm the patients. Transient deafness was observed in 1 patient, who recovered her hearing completely. It is worth noting that the reappearance of her ability to hear was concomitant with the administration of pyridoxine. Another toxic effect from vancomycin was a sudden hard shaking chill

TABLE XVII

Summary of Laboratory Work of Case 10

Date	Daily dosage of vancomycin, Gm.	Nonprotein nitrogen, mg./100 ml.	Urine output, ml.
March 13	2	50	
March 14	2		
March 15	2		
March 16	2	54	
March 17	2		
March 18	2		
March 19	2		
March 20	2		
March 21	2		
March 22	2		
March 23	2		
March 24	2	69	
March 25	2	74	
March 26	2		
March 27		84	1075
March 28			1225
March 29			1250
March 30			1350
March 31		72	625
April 2			300
April 3		104	200
April 4			200
April 6		142	250
April 7		107	
April 8			Died

and fever that developed during or immediately after the infusion. This we have observed in 2 cases in this series and in at least five other instances and it appears to be dangerous only if the patient is too sick to be able to stand the added fever, chills, and generalized reaction.

KANAMYCIN. Forty-five of the 50 patients treated with kanamycin suffered no overt toxicity. Three patients had evidence of severe nephrotoxicity, and in these patients the toxic reaction contributed to death.

CASE 11. The patient was a 60 year old white man who entered the hospital because of a progressive pneumonic process in the right lower lobe of the lung. He was given kanamycin intramuscularly, 0.5 Gm. four times daily, for 14 days. The blood nonprotein nitrogen concentration on admission was 30 mg./100 ml. It had risen to 68 mg./100 ml. on the thirteenth day of therapy and to 120 mg./100 ml. by 18 days after therapy was begun. The patient died of uremia 22 days after the start of therapy. Urinalysis on admission showed no casts or albumin and a urinalysis on the fourth day of therapy showed casts but no albumin.

CASE 12. The patient was a 70 year old white woman with diabetes who entered the hospital with *Escherichia coli* and staphylococcal bacteremia. She made an initial response to penicillin and chloramphenicol, but fever returned and she was given kanamycin, 0.5 Gm. intramuscularly four times daily, for six days. She then had a progressive rise in blood urea nitrogen concentration and a fall in urine output. She died of uremia and oliguria 19 days after kanamycin therapy was begun. The infection apparently was under control. Autopsy showed severe toxic nephrosis similar to that seen with neomycin.²⁰ (Table XVIII.)

CASE 13. The patient was an 87 year old woman with staphylococcal bacteremia and multiple abscesses. The bacteremia apparently responded to seven days of therapy with kanamycin given intravenously, 1 Gm. in 500 ml. of 5 per cent dextrose in water twice daily.

TABLE XVIII

Summary of Laboratory Studies on Case 12

Date	Dosage of kanamycin, Gm./day	Nonprotein nitrogen, mg./100 ml.	Urine output, ml.
Sept. 24	2	49	1000
Sept. 25	2		800
Sept. 26	2		N.R.*
Sept. 27	2		N.R.
Sept. 28	2		N.R.
Sept. 29	2		N.R.
Sept. 30	2		200
Oct. 1			N.R.
Oct. 2			400
Oct. 3			600
Oct. 4			N.R.
Oct. 6			400
Oct. 7			300
Oct. 8		178	30
Oct. 9		173	150
Oct. 10			400
Oct. 11			100

* Not recorded.

The blood nonprotein nitrogen concentration rose from 48 to 68 mg./100 ml. during therapy, but she died apparently in uremia and with oliguria on the seventh day of therapy. Unfortunately, blood chemistry determinations during the last five days of her life were not available. The kanamycin was given intravenously due to the fact that intramuscular injections were not possible because of skin abscesses.

One patient in this series* became completely deaf after receiving 18 Gm. of kanamycin over a nine day period.

CASE 14. The patient was a 68 year old woman with carcinomatosis and pyonephrosis due to obstruction, who was seen on Oct. 10, 1958, when the temperature rose suddenly to 106 F. She was given 6 Gm. of vancomycin during the next four days and had a clinical response and a marked lowering of the fever. Kanamycin was started in a dosage of 0.5 Gm. intramuscularly four times daily on October 14. On October 23 she complained of difficulty in hearing and within 48 hours became completely deaf. The kanamycin was discontinued, but she did not regain her hearing before she died suddenly 18 days later. Blood nonprotein nitrogen concentration was 50 mg./100 ml. at the start of the kanamycin therapy and 51 mg./100 ml. when it was discontinued 11 days later.

Toxicity experienced with kanamycin can be summarized by stating that this drug showed evidence of severe renal toxicity and that the patients it so affected were in the older age groups and acutely ill. It also caused 1 patient in this series to become deaf. In addition to the 3 fatal cases of renal toxicity we saw in this series, we have also seen a fatality from kanamycin in a 1 year old child who developed a urinary shutdown after 0.75 Gm. of kanamycin was given for staphylococcic bacteremia, and we saw another fatality due to kanamycin in a 48 year old diabetic patient who died with renal failure after a course of kanamycin given for a large staphylococcic abscess in the axillary region. These cases were not included in this series because they were not seen in alternation with the other

* We have seen 2 other cases of deafness from kanamycin in patients treated at the Veterans Hospital at Wood, Wis.

cases reported and their inclusion would have biased data on the incidence of toxicity. Many patients had transient rises in the nonprotein nitrogen concentrations, as shown in table V, but these were not considered major toxic reactions because the patients apparently suffered no harm from them.

DISCUSSION

This study is illustrative of the fact that the efficiency of an antimicrobial agent depends on the severity of illness of the patients to whom it is administered. In comparing antimicrobial agents, it is important to be sure that similar groups of patients are treated with each drug. Ordinarily, one is forced to do initial studies with new agents on patients who are not too seriously ill because there is no justification for investigating newer agents in seriously ill patients for whom proved therapy is available. This usually unavoidable situation has a tendency to make new drugs appear more effective than they might actually be because many of the less serious infections clear up on their own and the antibiotics being investigated are given the credit. The fact that the three agents that were studied were made available at the same time and that the patients treated had not responded to other antibiotics made it possible to do this comparative study with severely ill patients. The percentages of effectiveness of the three drugs were indeed comparable (ristocetin 33 per cent, vancomycin 39 per cent, and kanamycin 37 per cent). This is in spite of the fact that there are some differences between the drugs in regard to *in vitro* sensitivity of staphylococci to them and also in regard to the rates at which they kill staphylococci *in vitro*.⁹ At first glance, the rates of effectiveness of all three antibiotics may not seem too impressive, but it should not be forgotten that all the patients treated were acutely ill and had not responded to other antibiotics. Thus, these data do confirm the fact that ristocetin, vancomycin, and kanamycin all are powerful antimicrobial agents that undoubtedly have a place in our antimicrobial armamentarium. They also show that the problem of severe staphylococcal infections will probably need solutions other than those provided by antibiotics. This was particularly highlighted in this series of cases by 2 patients with staphylococcal endocarditis each of whom received penicillin, streptomycin, bacitracin, ristocetin, kanamycin, vancomycin, and novobiocin, all in maximum dosage, and who at postmortem yielded living staphylococci from the valvular vegetations. Table X illustrates the fact that the therapeutic rule of always changing therapy when the patient is not responding to treatment will in certain cases hold true with the use of these antibiotics, because in 5 of 17 severe cases in which this was done, the change of antibiotic was followed by a therapeutic response.

All three drugs demonstrated renal toxicity and ototoxicity and ristocetin in addition showed skin and bone marrow toxicity even though the total number of patients studied was relatively small.

In 5 patients of the 131 studied, the antibiotics were felt to be a contributing factor to death. Admittedly, the seriousness of the illnesses contributed to the instances of toxicity, but even with this reservation, these drugs are obviously more toxic than penicillin, the tetracyclines, chloramphenicol, and erythromycin which were given to many of the same patients. The only toxicity from these three older,

proved drugs was the development of staphylococcic diarrhea in 10 patients who had received the tetracyclines. For this reason we believe that when one of these other agents is active against staphylococci isolated from an infection, it remains the drug of choice. The one reservation to this would be in cases of staphylococcal endocarditis in which, if the organism is not penicillin sensitive, a relatively bactericidal agent such as neomycin, bacitracin, ristocetin, kanamycin, or vancomycin should be used. It should be remembered that bacterial endocarditis is the only disease in which bactericidal activity has been shown to be equated with clinical results, so with this one exception, in vitro bactericidal activity cannot be considered to give a drug a clinical advantage.²⁰ For instance, in staphylococcal bacteremia the so-called bacteriostatic agents were found to be just as effective as the bactericidal drugs when they were used against sensitive strains of staphylococci.²¹ In this connection it should be pointed out that in spite of the in vitro bactericidal activity of all three drugs studied, staphylococci were isolated with ease from patients who were receiving each of the three antibiotics (table IX).

The over-all toxicity of ristocetin, kanamycin, and vancomycin is very similar to that of neomycin, and the use of the former drugs probably will eventually follow the pattern of neomycin, which has generally been reserved for the most severe types of infections in which the risks of toxicity are justified.¹⁸

SUMMARY AND CONCLUSIONS

Ristocetin, vancomycin, and kanamycin were alternately administered to 131 patients with severe staphylococcic infections or bacterial endocarditis. The groups of patients receiving each drug were found to be comparable, and the relative clinical effectiveness and toxicity of the three drugs were determined. Each drug was effective in slightly more than one third of the patients studied. No one antibiotic showed marked clinical superiority over the others.

Significant ototoxicity and nephrotoxicity were found with ristocetin, kanamycin, and vancomycin. In addition, ristocetin caused a high incidence of skin reactions and caused depression of the granulocytic series of white blood cells in some patients.

The conclusion was reached that ristocetin, kanamycin, and vancomycin are effective antistaphylococcal agents of approximately comparable activity. They all exhibit toxicity greater than that found with the tetracyclines, penicillin, chloramphenicol, and erythromycin. Therefore, ristocetin, vancomycin, and kanamycin should be used only for patients who have not responded or are not likely to respond to safer antibiotics and when the condition of the patient justifies the risk of serious toxic reactions.

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Preliminary Evaluation of the Incidence of Reactions to Penicillin

W. J. BROWN, W. G. SIMPSON, AND ELEANOR V. PRICE

Venereal Disease Branch, Communicable Disease Center, Atlanta, Ga.

Each year well over a quarter of a million patients are treated for syphilis or gonorrhea in public health facilities in the United States. Penicillin is not only the drug of choice for the treatment of these diseases, but in terms of efficacy and economy, there is no adequate substitute. Reported increases in the incidence and severity of penicillin reactions are therefore of major concern to those charged with the control of venereal disease.

Such reports instigated a study of penicillin reactions among venereal disease clinic patients, which was conducted by the Public Health Service in 1954.¹ The initial study, which covered the period from April 15 to Aug. 15, 1954, was later extended so that a larger sample of patients treated with benzathine penicillin G might be included. Records were received on a total of 19,510 patients, 116 of whom were reported as experiencing reactions to penicillin therapy. The incidence of reactions, which was 6/1000 total cases treated, varied from 3/1000 treated in single session therapy to 56/1000 treated with schedules of two or more weeks in duration. Since the longer treatment schedules afforded greater opportunity for reactions to be observed, it was felt that the rate of 56/1000 was nearer the true incidence of reactions in the series.

During the five years that have elapsed since 1954, there has been an increasing awareness of penicillin sensitivity among venereal disease clinicians, particularly those unfortunate enough to have encountered severe anaphylactoid reactions and, in some rare instances, death from penicillin treatment. This situation prompted a re-evaluation of the incidence of reactions among venereal disease patients.

This is a preliminary report of the current evaluation, which was initiated in March, 1959. The present study has been conducted in the same manner as the 1954 study, i.e., the participating treatment facilities were requested to submit a card bearing the race, sex, age, diagnosis, history of previous penicillin treatment, and planned treatment schedule for each patient treated with penicillin during the course of the study. No special follow-up was requested, but the participants were asked to instruct patients to report to the clinic if any discomfort attributable to the treatment was experienced. It was realized that many minor reactions would be missed, but it was assumed that most patients would return if severe reactions occurred. The single departure from the previous procedure was a request, as a safeguard, that patients be detained in the clinic, if possible, for a 30 minute period after treatment.

Records have been received to date on a total of 22,853 patients treated with

This study was made possible through the cooperation of the Departments of Health of Alabama, Arkansas, Chicago (Illinois), Detroit (Michigan), District of Columbia, Florida, Georgia, Kansas, Kentucky, Mississippi, Missouri, New Mexico, North Carolina, Oklahoma, Oregon, Philadelphia (Pennsylvania), Puerto Rico, South Carolina, Tennessee, Washington, and West Virginia.

TABLE I
Comparative Frequency of Reactions to Penicillin in 1959 and 1954

	1959 study		1954 study	
	Number	Rate per 1000	Number	Rate per 1000
Total patients treated	22,853		19,510	
Total patients reacting	218	9.54*	116	5.95
Urticaria	125	5.47	96	4.92
Anaphylaxis	20	0.88*	4	0.21
Moderate to severe	7	0.31	4	0.21
Mild	13	0.57*		
Serum sickness	10	0.44	5	0.26
Other reactions				
Pruritus, generalized	19	0.83*	2	0.10
Vertigo, syncope	18	0.79*	—	0.00
Gastrointestinal upset (nausea, vomiting, abdominal pain).	14	0.61*	1	0.05
Chills, fever, headache	9	0.39*	—	0.00
Angioneurotic edema	5	0.22	—	0.00
Dermatitis medicamentosa	4	0.18	5	0.26
Chest pain, dyspnea	2	0.09	2	0.10
Erythema multiforme	1	0.04	1	0.05
Dermatophytid	1	0.04	1	0.05
Hysteria	1	0.04	—	0.00

* Difference between 1959 and 1954 significant at the 5 per cent level.

penicillin. Reactions were observed in 218, or 9.54/1000 treated—a rate significantly greater at the 1 per cent level than the rate of 5.95/1000 observed in 1954.

A comparison of the incidence of various types of reactions observed in the two studies is presented in table I. Differences between the two that are statistically significant at the 5 per cent level have been indicated by an asterisk on the higher rate.

Clinicians were requested to classify reactions as urticaria, anaphylaxis, serum sickness, or other. Except for mild anaphylactic reactions, the difference in incidence of the three major categories of reactions for 1959 and 1954 is no greater than could be attributed to chance. It is believed that detaining the patients for 30 minutes after treatment accounted not only for the observation of the mild anaphylactic reactions in 1959, but for such symptoms as nausea and vomiting, vertigo, and syncope, which were relatively rare in 1954. In general, these represent complaints that could have been dismissed by patients as too inconsequential to report. Further evidence that the apparent increase in incidence of reactions is at least partly the result of detaining the patients is the fact that 51 per cent of reactions reported in 1959, but only 16 per cent of those reported in 1954, occurred on the first day of treatment.

The reactions classified by clinicians as "other" were not associated with urticaria, anaphylaxis, or serum sickness and therefore do not represent the frequency of occurrence of such symptoms. For example, 77 patients complained of generalized pruritus, but in all but 19 instances this was associated with urticaria. Figure 1 shows the relative frequency with which various complaints were listed.

Urticaria and generalized pruritus were by far the chief complaints. Angioneurotic edema was next, followed closely by nausea and vomiting, syncope, vertigo,



FIG. 1. The relative frequency of complaints following penicillin therapy.

and chills and fever. Encountered less frequently but of greater importance are such symptoms as abdominal cramps, dyspnea, hypotension, and chest pain, which were associated, in general, with anaphylaxis.

A sample composed of the first 14,000 reports received has been tabulated by factors that the 1954 study indicated influenced the incidence of reactions. The cases have been divided between single session and multiple injection schedules since the opportunity for observing reactions was greater in one group than in the other.

Table II shows the incidence of reactions by type of penicillin, total amount of penicillin in planned schedules, and diagnosis. Among patients treated on single session schedules, the highest reaction rate followed the administration of benzathine penicillin G. It will be observed, however, that this difference disappears in multiple injection schedules. Furthermore, single session schedules of 2,400,000 to 4,800,000 units produced significantly more reactions than schedules of less than 2,400,000 units. The large dosage schedule of 2,400,000 to 4,800,000 units in a single session was used in 31 per cent of the patients treated with benzathine penicillin, 11 per cent of those on a combined schedule, and only 2 per cent of those treated with procaine penicillin G in oil with 2 per cent aluminum monostearate.

In comparing single session with multiple injection schedules, it would appear that single session schedules produce more reactions than the same amount administered in divided doses. The differences, however, are not statistically significant. The single session schedules measure the effect of varying amounts of treatment since the treatment indicated was received. In the multiple injection schedules,

TABLE II
Incidence of Penicillin Reactions in Sample of 14,065 Cases

	Single session schedules			Multiple injection schedules		
	Patients treated	Patients reacting		Patients treated	Patients reacting	
		Number	Rate per 1000		Number	Rate per 1000
Type of penicillin						
Procaine penicillin G in oil	4942	29	5.9	1225	38	31.0
Benzathine penicillin G	2209	23	10.4	677	19	28.1
Procaine and benzathine	4645	22	4.7	73	3	41.1
Aqueous procaine	167	1	6.0	112	3	26.8
Planned schedule, units						
Less than 1,200,000	1624	12	7.4			
1,200,000-2,400,000	9060	42	4.6	264	—	0.0
2,400,000-4,800,000	1286	21	16.3	538	4	7.4
4,800,000-6,000,000				1074	47	16.3
6,000,000 or more				200	12	60.0
Diagnosis						
Epidemiological treatment	3342	19	5.7	298	3	10.1
Gonorrhea	8244	44	5.3	482	2	4.2
Syphilis	316	9	28.5	1309	58	44.3

with the incidence of reactions varying from 7.4 to 60.0/1000 treated, the planned schedule is shown (table II). Actually, 88 per cent of the patients who reacted to planned schedules of 4,800,000 units or more reacted before treatment had been completed. The multiple injection schedules also reflect the greater opportunity for reactions to be observed—the larger dosage schedules, in general, requiring a longer period to administer.

Patients treated for syphilis experienced the highest incidence of reactions. It is

TABLE III
Incidence of Penicillin Reactions in Sample of 14,065 Cases

	Single session schedules			Multiple injection schedules		
	Patients treated	Patients reacting		Patients treated	Patients reacting	
		Number	Rate per 1000		Number	Rate per 1000
History of penicillin treatment						
No previous penicillin	1,242	4	3.2	269	6	22.3
Previous penicillin						
Did not react	10,282	60	5.8	1728	49	28.4
Reacted	86	6	69.8	18	5	277.8
Race and sex						
White Male	629	5	7.9	103	5	48.5
White Female	442	3	6.8	123	5	40.7
Negro Male	6,222	33	5.3	534	15	28.1
Negro Female	4,486	33	7.4	572	26	45.5
Age, years						
10-19	2,704	11	4.1	327	1	3.1
20-29	6,062	40	6.6	679	13	19.2
30-39	2,194	15	6.8	392	14	35.7
40-49	574	8	13.9	283	14	49.5
50 and older	220	1	4.5	377	21	55.7

believed, however, that this is attributable to the larger dosage schedules administered for this disease and to better follow-up of syphilitic patients. Although the reaction rate of 28.5 for single injection therapy appears out of line with the rate of 16.3 after treatment with 2,400,000 to 4,800,000 units, the single session schedule with which most syphilitic patients were treated, the difference between the two is not significant.

The effect of previous penicillin, race, sex, and age on the incidence of reactions is presented in table III. It is the policy in most venereal disease clinics to use other drugs in treating patients who give a history of sensitivity to penicillin. This accounts for the small number of such patients included in this series. The incidence of reactions in this small group was high—11 of the 104 experiencing further reactions. In our 1954 study it appeared that patients who had tolerated previous penicillin were less likely to react than patients who had never received penicillin therapy. In this series the differences between 5.8 and 3.2/1000 for single session therapy and between 28.4 and 22.3/1000 for multiple injection schedules (differences that are in the opposite direction) are not statistically significant.

Race and sex differences, which were noted in our earlier study, are not apparent in this sample. Age, however, is a factor, with the young patients tolerating treatment better than the old.

To date, no deaths have occurred during the period covered by the study. Emphasis has been placed on preparedness, and all clinicians have been advised to keep emergency drugs and equipment in readiness in all treatment areas so that fatal reactions may be averted.

SUMMARY AND CONCLUSIONS

The preliminary results of an evaluation of reactions to penicillin therapy presently being conducted by the Public Health Service in venereal disease clinics indicate that the incidence of reactions is somewhat greater now than in 1954. It is believed, however, that the increase is more apparent than real and may be attributed in large part to the present practice of detaining patients in the clinic for a 30 minute period after treatment. This is substantiated by the time at which the reactions were noted and the difference in the types of reactions reported. If there is an actual increase, it is of insufficient magnitude to warrant a change in present treatment procedures.

It is not our intent, however, to minimize the seriousness of the problem in venereal disease control. Since a high percentage of patients in venereal disease clinics have previously received the same treatment, there is a steady reduction in the number who are penicillin tolerant and a constant increase in the number who are penicillin sensitive and are treated with other drugs. Records from one clinic indicate that the penicillin-sensitive group already has reached the proportion of 10 per cent of clinic admissions.

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Influence of Chemical Structure Upon Biological Properties of Penicillin

G. H. BERRYMAN AND J. C. SYLVESTER

Abbott Laboratories, North Chicago, Ill.

In terms of chemical structure, the various penicillins contain a moiety that is common to them all and a side chain that differs and thereby confers individuality. A seemingly different antibiotic, synnematin, can also be viewed as a member of the penicillin family because it, too, contains the common moiety (6-amino penicillanic acid). On the other hand, it has an entirely different origin,* and it is characterized by a straight side chain. This D-amino acid aliphatic structure contrasts sharply with the *cyclic* structure found in the side chains of other penicillins. The comparative structures of penicillin G, penicillin V, and synnematin are shown in figure 1.

We may ask—what differences in biological properties result from this structural departure?

EFFECT UPON ANTIGENICITY

To determine if there are differences in the capacities of synnematin and penicillin G to function as antigens, passive transfer studies were carried out. In this procedure, local skin sites of nonallergic recipients are passively sensitized by intracutaneous administration of serum that has been obtained from patients recovering from a recent penicillin reaction. A certain proportion of such sera contain reagins against the penicillin causing the reaction. For all intents and purposes this is either penicillin G or penicillin V, which are the only two penicillins in common clinical use. When the offending penicillin is subsequently introduced into the passively sensitized areas of the recipient's skin, a local whealing reaction is obtained because of the specific antigen-antibody union at that site. This is the Prausnitz-Kustner reaction, of long existence and familiarity to investigators in the fields of allergy and immunology.

Upon challenging these passively sensitized sites with either synnematin or penicillin G or V, it was found that synnematin did not produce a positive local reaction but, as expected, both penicillins G and V did.

Confirmatory studies were then carried out by direct intradermal skin tests in 2 penicillin-sensitive patients. The results are shown in table I. Markedly positive skin reactions were obtained to penicillins G and V but not to synnematin. For additional details, the reader is referred to a previous report that deals with the antigenic aspects at length.¹

Data have recently appeared describing the safe use of synnematin in penicillin-sensitive patients who required treatment for gonorrhea or syphilis.²

* Synnematin is derived not from a *Penicillium* species but from a mold classified as *Cephalosporium salmosynnematin*.

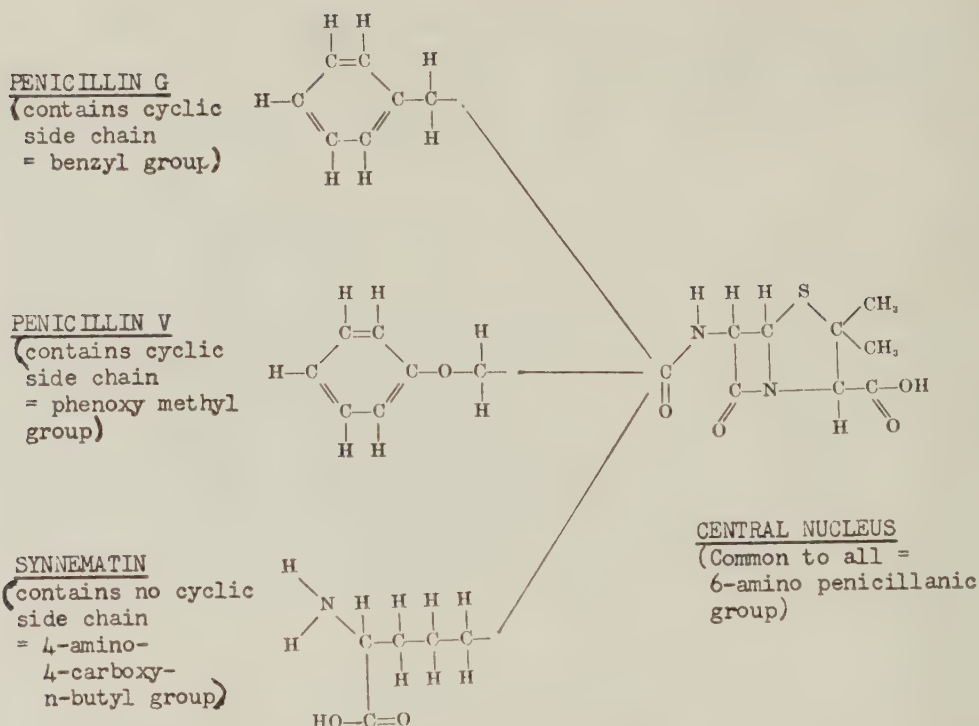


FIG. 1. Comparative structures of penicillin G, penicillin V, and synnematin.

EFFECT UPON ANTIBIOTIC ACTIVITY

It has been established previously that synnematin is unique among penicillins for its activity against certain gram-negative organisms.^{3,4} For the present report, comparative studies were carried out to determine the side by side effectiveness of synnematin and penicillin G against selected gram-negative and gram-positive organisms. In table II the comparative sensitivities to these two antibiotics are shown for seven strains of coagulase-positive staphylococci. Only one of these strains (MIH 8) exhibited innate or natural sensitivity to penicillin G. The others are by nature penicillin-resistant. The data indicate that while penicillin G was approximately 160 times as active as synnematin against the penicillin-sensitive strain, yet the difference in activity against the resistant strains, which produce penicillinase, was much less pronounced; indeed, synnematin may have been slightly more active than penicillin G in some cases.

TABLE I

*Skin Reactions (cm.) from Direct Intradermal Tests**

Drug	After ½ hour		After 1½ hours	
Penicillin G	W 1.4	E 2.5	W 1.4	E 2.5
Penicillin V	W 2.2	E 2.2	W 5.0	E 5.0
Synnematin	0		0	
Control saline	0		0	

* W = wheal; E = erythema.

TABLE II

Comparative Activity* of Synnematin and Penicillin G Against *Staphylococci*

Strain	Minimum inhibitory concentration, µg./ml.	
	Synnematin	Penicillin G
<i>Staphylococcus</i> MIH 8†	12.0	.075
<i>Staphylococcus</i> MI 67 PR	>154.0	154.0
<i>Staphylococcus</i> MIH 2	24.0	38.0
<i>Staphylococcus</i> MIH 5	96.0	154.0
<i>Staphylococcus</i> Columbia U. 6112381	600.0	>240.0
<i>Staphylococcus</i> Columbia U. 801538	>600.0	>240.0
<i>Staphylococcus</i> Columbia U. 032723	600.0	>240.0

* The minimum inhibitory concentrations of the two antibiotics were determined by a serial tube dilution procedure employing tryptose phosphate broth. The inoculum in each tube was 0.1 ml. of a 1:10 dilution of an 18 to 20 hour broth culture of the test strain. The tubes were incubated at 37 C. and read after 48 hours' incubation.

† This strain has an inborn sensitivity to penicillin G; the remaining six strains are naturally resistant.

In table III are summarized the comparative activities of synnematin and penicillin G found against a variety of different microorganisms. It is apparent that the antimicrobial spectra of synnematin and penicillin G are significantly different. Synnematin is much less active than penicillin G against gram-positive organisms. Against gram-negative organisms, however, synnematin has equivalent or somewhat higher activity than penicillin G. This greater activity of synnematin is particularly well manifested in the case of *Salmonella*.

It is evident from these data that the microbiological properties of synnematin are distinctly different from those of penicillin. This has been reflected clinically in the successful use of synnematin against typhoid infections in Mexico⁵ and the

TABLE III

Comparative Antibacterial Activity* of Synnematin and Penicillin G

Organism	Minimum inhibitory concentration, µg./ml.	
	Synnematin	Penicillin G
<i>Aerobacter aerogenes</i> ATCC 129	12.5	3.1
<i>Diplococcus pneumoniae</i>	3.1	0.02
<i>Enterococcus</i> 89	100.0	3.1
<i>Escherichia coli</i> Juhl	100.0	25.0
<i>Proteus mirabilis</i> Finland 9	3.1	50.0
<i>Proteus vulgaris</i> Abbott J. J.	400.0	400.0
<i>Pasteurella multocida</i> ATCC 10544	0.39	0.04
<i>Salmonella typhosa</i> ATCC 9992	1.6	9.3
<i>Salmonella typhimurium</i> Edwards 9	1.6	6.2
<i>Shigella sonnei</i> ATCC 9290	100.0	50.0
<i>Staphylococcus aureus</i> MI-45	6.2	0.04
<i>Streptococcus pyogenes</i> ATCC 8668	1.6	0.005

* The minimum inhibitory concentrations of the two antibiotics were determined by a serial tube dilution procedure employing tryptose phosphate broth. The inoculum in each tube was 0.1 ml. of a 1:10 dilution of an 18 to 20 hour broth culture of the test strain. The tubes were incubated at 37 C. and read after 48 hours' incubation.

United States.⁶ It is interesting to recall that while penicillin G has also been reported to have some effectiveness in typhoid fever, special measures to produce extremely high doses were deemed necessary to insure benefit.⁷

A different facet of the comparative antibacterial actions of synnematin and penicillin G has been described in relation to their single and combined effects.⁸ Judging from the inhibitory ratio of each antibiotic used individually versus the inhibitory ratio of the two used in combination, the two agents together demonstrate synergism against a resistant strain of *Micrococcus pyogenes*. Further, the development of resistance toward the individual antibiotics, seen after the third transfer of a sensitive strain of *M. pyogenes*, was not observed with the combination. The general conclusion was reached that "the two antibiotics may have somewhat different sites of action upon a single strain, as well as different effectiveness against different species." It was speculated that the amino acid type of side chain in synnematin would make this agent especially effective against the cell walls of certain specific bacteria which are not affected by benzyl penicillin.

OTHER BIOLOGICAL PROPERTIES

Substrate for Penicillinase. The lack of antibacterial activity exhibited by both synnematin and penicillin G against bacteria that contain penicillinase is to be expected because both antibiotics contain the β -lactam ring, the site of enzymatic degradation. Yet it appears that so specific a reaction as enzyme-substrate can be caused to change to a degree by the side chain difference. Although rates of inactivation of synnematin and penicillin G are similar in an acid medium (pH 3, 37 C.), the rate for the former was only one fourth that of the latter when both were exposed to an exocellular penicillinase derived from *Proteus vulgaris*.⁹

The explanation for these findings may extend beyond the differences in chemical configuration and could possibly be related to the differences in spatial relationships, which are brought about by the modification of the side chain. If one can draw the analogy between the "key in lock" and enzyme-substrate action, it would seem likely that a change in side chain should result in differences in rate of enzyme-substrate reaction. While it may have no direct relationship, it has been observed that synnematin is the only penicillin-like substance capable of forming a zwitterion, because of the amino and carboxyl groups of its side chain.¹ As a result, the accessibility of the substrate to the enzyme may be changed.

Absorption from Gastrointestinal Tract. Synnematin has been shown to be less absorbable to the human than penicillin G following oral administration. Oral doses of synnematin as high as 1.6 *M* units produced no detectable blood level.⁹ It would appear, therefore, that this biological property is also related to the nature of the side chain, judging not only by comparison of the oral absorbabilities of synnematin versus penicillin G, but also from the change in absorbability found with penicillin V over penicillin G. Here, substitution of the phenoxymethyl group in penicillin V for the benzyl group of penicillin G also effects a change in the absorption rate of those two penicillins.

SUMMARY

Although synnematin and penicillin G are derived from different biological

sources, their chemical structures are related to the "parent amine," 6-aminopenicillanic acid. Their chemical differences occur only in the side chain, where an aminoacyl structure (synnematin) contrasts with an aracyl structure (penicillin G). These chemical differences result in marked changes in the respective antigenic, antibacterial, and absorption characteristics of these two antibiotics. The physicochemical and spatial characteristics conferred by the D-amino acid side chain versus the phenylacetic acid side chain play an important part in determining the biological capacities of these antibiotics.

These conclusions are supported by data obtained from skin testing procedures in penicillin-sensitive patients, by *in vitro* studies with representative bacteria, and by the differing areas of clinical usefulness for the two antibiotics.

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Effect of Local Penicillin Spray on Survival Time Following a Massive Open Wound

F. B. BRINKLEY, R. F. SCHNEIDER, A. M. STEVENS, AND D. LINDSEY

Traumatic Investigation Branch, Biophysics Division, Chemical Warfare Laboratories, Army Chemical Center, Md.

During the past several years, intensive studies have been made in these laboratories of the mechanism of death following massive open wounds of nonvital areas. Recent reports by Wise et al.^{1,2} and Lindsey³ have summarized the work and have clarified the mechanism of death to the extent of demonstrating that shock and wound infection are together the major determinants of survival time.

Increasing interest in the specter of casualties *en masse*—as a result either of war or natural disaster—has pointed up the real significance of certain valid medical aims that are not nearly so prominent in the conventional practice of medicine. To emphasize the salvage of life, even at the expense of limb, function, or appearance, is no radical departure. To consider deliberate simplification of treatment, at some compromise in absolute quality, is somewhat more unconventional. To study an experimental treatment that is designed to permit temporization and delay is distinctly not the usual approach. Yet, in mass casualty situations, temporizing—and simplified temporizing—may be enforced by the circumstances and may offer a valuable means of saving human life.

Knecht et al.^{5,6} recently demonstrated in these laboratories that a bacitracin-neomycin-detergent solution in conjunction with intramuscular penicillin, sprayed over the damaged tissues immediately after wounding, is of highly significant effectiveness in prolonging survival time (mean survival was 100.47 hours) in our wound preparation. Use of the spray permits considerable delay in definitive surgical treatment; successful débridement can be carried out as late as 24 hours after wounding.

In view of the relatively greater availability of penicillin, it seemed indicative to study the effectiveness of immediate and delayed penicillin spray treatment on survival time following a massive open wound.

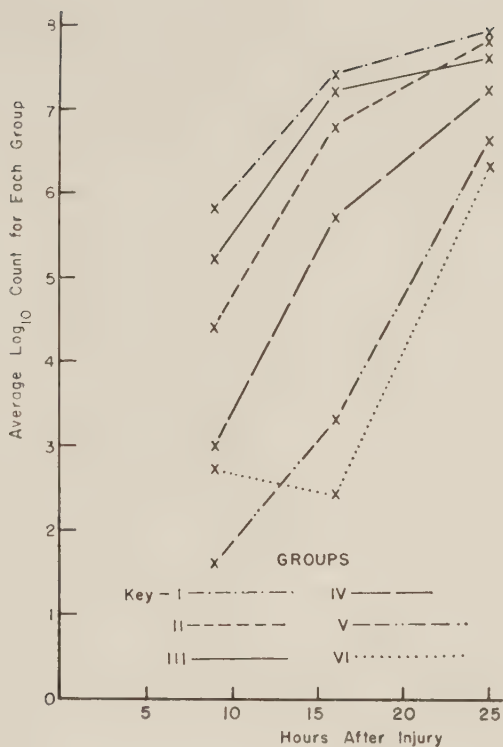
EXPERIMENTAL

Wound Preparation. Texas Angora goats (castrated males) weighing 35 to 50 Kg. were wounded in the manner described by Ochsner et al.⁴ As in the recent studies of Knecht et al.,^{5,6} the wounds were unilateral. Detonation of high explosive charge on the surface of the thigh produces massive destruction of both the quadriceps and hamstring muscle groups and comminution of the femur from the greater trochanter to the condyles.

Treatment. Animals were assigned at random to experimental groups, and simultaneous comparisons were made between all groups.

GROUP I. No treatment except for the routine initial hemostasis and dressing; the animals were left undisturbed until death (9 animals).

FIG. 1. Bacterial count for each group of animals.



GROUP II. Treatment was limited to local normal saline solution spray. Five hours after wounding the dressing was removed and the animal was manipulated so the hindquarters hung over the edge of the cart. A single application of 25 ml. of normal saline solution was sprayed into the wound after the manner of Knecht (9 animals).

GROUP III. Similar to group II, except that immediately following the wounding and before dressing the wound received treatment with the local saline solution spray. The spray therapy was repeated every eight hours thereafter for a 24 hour period (9 animals).

GROUP IV. Intramuscular penicillin (1.2 Gm. crystalline potassium penicillin G) immediately following wounding and every eight hours thereafter for a 24 hour period. The animals were otherwise left undisturbed until death (9 animals).

GROUP V. These animals were treated with the same spray therapy as in group III. However, the spray solution contained 1.2 Gm. (2,000,000 units) of crystalline potassium penicillin G (9 animals).

GROUP VI. Identical to group II, except that the spray solution contained 1.2 Gm. of penicillin (9 animals). The effect of manipulation of the animal upon survival time has been reported by Ochsner et al.⁴

Observations. Survival times were recorded. Serial bacteriological samples of the wound exudate were taken at 9, 16, and 25 hours after wounding. Quantitative estimations of clostridial concentration were made by the methods of Lindsey et al.^{7,8} A standard dairy loop that delivers 0.01 ml. was used to streak the surface of the medium in standard Petri dishes. Samples of the exudates were taken with standard cotton tip swabs which delivered approximately 0.1 ml. of material. The

TABLE I

Clostridial Concentration in Wound Exudate*

Group	Treatment	Time after wounding, hr.		
		9	16	25
I	None	5.8	7.4	7.9
II	Local saline, delayed, single application	4.4	6.8	7.8
III	Local saline, repeated	5.2	7.2	7.6
IV	Intramuscular penicillin, repeated	3.0	5.7	7.2
V	Local penicillin, repeated	1.6	3.3	6.6
VI	Local penicillin, delayed, single application	2.7	2.4	6.3

* Concentration: mean \log_{10} of *Clostridium perfringens* per ml. of wound exudate; plate estimations on blood-azide egg-yolk medium.

swabs were placed in 0.9 ml. of cold sterile Trypticase soy broth and used as the initial dilution (10^{-1}). The loop was then used to transfer 0.01 ml. (10^{-3} dilution) to the surface of the agar. Systematic streaking of the surface yielded subsequent dilutions up to 10^{-8} .

RESULTS

Bacterial growth curves are presented in figure 1, and show the effect of penicillin intramuscularly and/or locally on the flora of the wound. The application of local saline had little effect on the wound flora (table I) and the survival (table II).

The mean survival of each group of animals is recorded in table II and shown graphically in figure 2.

TABLE II

Survival Time Following Unilateral Wounding

Group	Treatment	Survival time, hr.*
I	None	25.6 \pm 0.88
II	Local saline, delayed, single application	29.5 \pm 1.65
III	Local saline, repeated	33.6 \pm 2.37
IV	Intramuscular penicillin, repeated	55.5 \pm 16.76
V	Local penicillin, repeated	57.7 \pm 2.79
VI	Local penicillin, delayed, single application	63.3 \pm 7.50

* Survival time: mean survival time, and standard error of mean, for groups of 9 animals each.

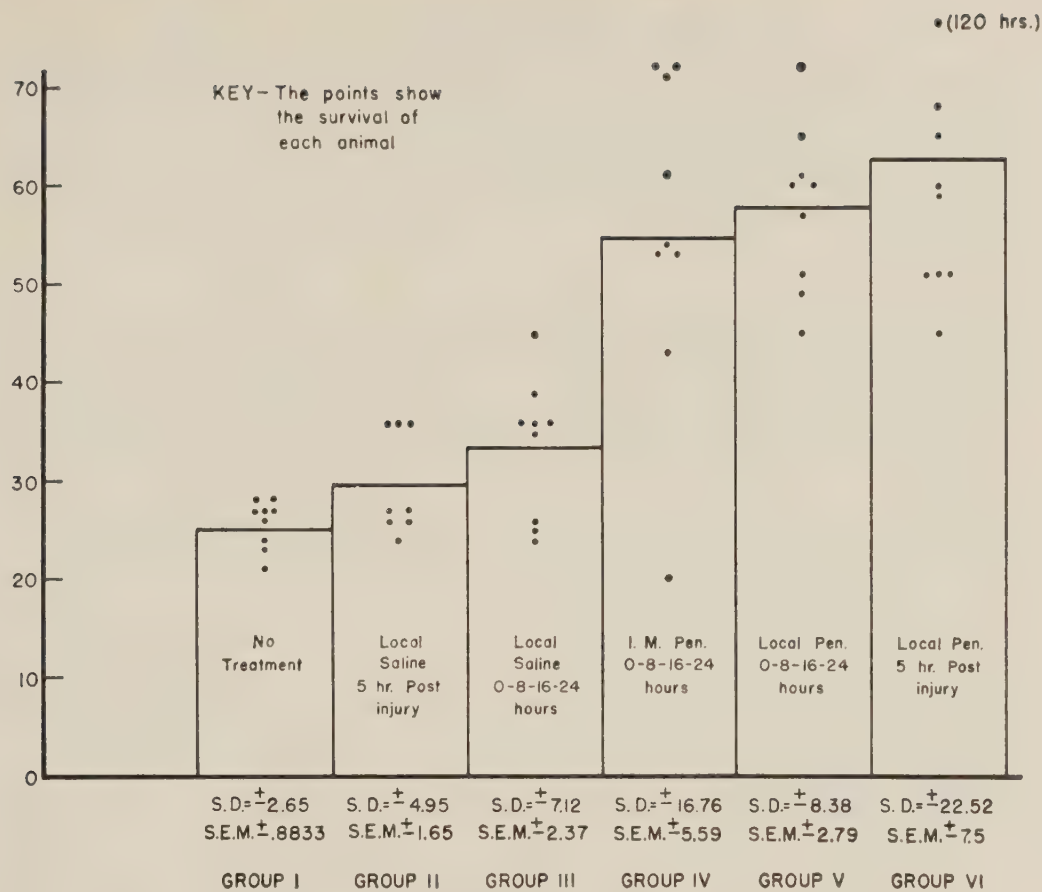


FIG. 2. Average survival times of the six groups of animals.

DISCUSSION

It is evident from the data in figures 1 and 2 that the local application of a penicillin spray, be it five hours postinjury or immediately after injury and every eight hours thereafter over a period of 24 hours, is of the same order of effectiveness as the intramuscular administration of the same amount of penicillin.

The fact that the penicillin spray is effective, combined with the simplicity of administration, adds up to an implication of some importance in the treatment of wounded under mass casualty conditions.

Local chemotherapy of surgical infections, including war wounds, has been investigated employing the majority of antibacterial agents available. Parenteral penicillin in conjunction with local penicillin and/or local sulfonamides was employed with limited success during World War II and the Korean conflict.^{9 11}

The penicillin spray is of highly significant effectiveness in comparison to no treatment at all or the application of saline alone. Although penicillin may not be the optimum antibiotic for battlefield use, it is the most effective single component so far tested as a spray. It is readily available, and it is probable that it would find ready professional acceptance as an item for use by untrained or hastily trained medical assistants.

CONCLUSIONS

In a lethal open wound in the goat, penicillin administered locally by spray is effective in suppressing the significant bacterial flora and in prolonging survival. The effect of intramuscular penicillin in liberal doses is no more effective than local penicillin administered five hours postinjury or immediately after injury and every eight hours thereafter over a period of 24 hours.

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Untoward Reactions in Human Beings from Application of Antibiotics in Plant Disease Control

HERBERT S. GOLDBERG, JOHN T. LOGUE, AND R. N. GOODMAN

School of Medicine and College of Agriculture, University of Missouri, Columbia, Mo.

Antibiotics for plant disease control are presently available in several commercial preparations. Among the antibiotics most frequently applied to plants are streptomycin and cycloheximide. The latter is used to control some half dozen fungal diseases of plants, such as cherry leaf spot, powdery mildew of wheat, and various turf diseases.^{1,2} Streptomycin is used to control many bacterial diseases of plants, such as bacterial spots of tomato, pepper, and peach, and bacterial blights of pear, apple, tomato, and celery.^{1,2}

As a result of this plant disease control activity, these antibiotics are applied rather frequently in some of the larger fruit- and vegetable-growing areas of the United States.

Since both of these antibiotics are known to have caused untoward reactions in human beings when used chemotherapeutically in medical problems,^{3,4} interest was aroused in any potential hazards these compounds might present to field workers utilizing the materials in plant disease control. Indeed, it has been demonstrated that allergic reactions to streptomycin have often been of more concern to nurses and others administering the antibiotics than to the patients receiving it.⁵

Accordingly, an investigation was carried out to evaluate potential hazards to persons applying antibiotics in plant disease control.

MATERIALS AND METHODS

With the aid of county agents and farm advisors, two geographical areas of the United States were included in this study. The major fruit- and vegetable-raising sections of Missouri and the walnut- and pear-growing areas of northern California (150 to 200 miles radius from San Francisco) provided test subjects for this project (figs. 1 and 2).

The major test for development of an untoward reaction was an intradermal tuberculin-type skin test. The survey procedure consisted of going directly into the various orchards in the midst of daily activity and skin testing the field hands on the spot. A total of 80 persons applying streptomycin regularly over a three to four year period (3 to 10 sprays/season) were skin tested for hypersensitivity using 500 µg. streptomycin in 0.1 ml. sterile water, with suitable controls injected in opposite arms.⁶ An equal number of randomly selected adults, agricultural and nonagricultural workers, not previously exposed to streptomycin in any known form, served as a control skin-testing group. Tests were read after 48 hours for erythema and wheal at the site of streptomycin injection with comparison to readings at the control site (no antibiotic).

In addition to the skin testing, all exposed persons were queried for reported



FIG. 1. Shaded areas indicate counties in California where testing and evaluating were carried out.

reactions during antibiotic application and were observed during daily spray activity.

Supplementary records were kept for cycloheximide users with the exception of the skin test, since cycloheximide is known to be a skin irritant.⁷

RESULTS

Of the 80 field workers exposed to streptomycin in their work, only 2 gave a positive tuberculin-type skin test to streptomycin injection. Of 75 randomly selected persons skin tested in the same manner, 3 gave a positive test.

In addition, 2 young adult Japanese field workers developed repeated contact dermatitis on wrists and neck during application of antibiotic spray. However, these persons failed to demonstrate a positive intradermal test for streptomycin sensitivity.

All obtainable data from cycloheximide users indicated no untoward reactions with one notable exception. This case is described.

A 50 year old male agricultural worker was seen immediately after he had spent three hours spraying mushrooms with a spray containing 2.6 parts million cycloheximide for fungal disease control. These mushrooms were grown in large trays, stacked six in a unit with 24

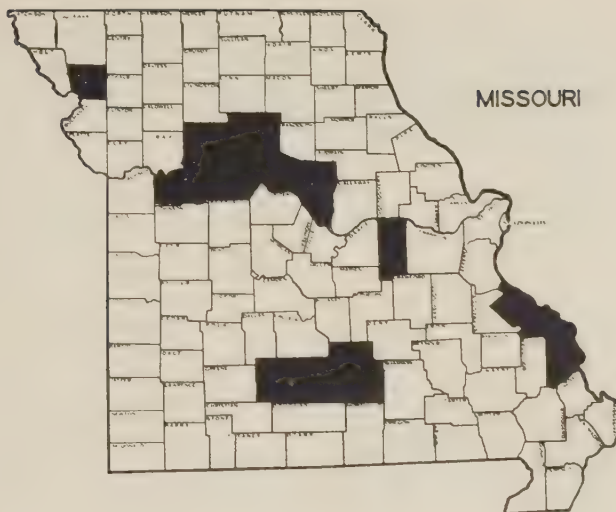


FIG. 2. Shaded areas indicate counties in Missouri where testing and evaluating were carried out.

inches of clearance between trays, and maintained at optimal temperature and humidity in dry underground caves. Figure 3 illustrates the arrangement and handling of these mushroom beds as well as the problem of spray administration.

As a result of this arrangement, cycloheximide spray was inhaled freely and soaked through the patient's shirt. The patient developed nausea, vomiting, recurrent abdominal cramping, and loose, watery diarrhea. These symptoms subsided without specific treatment to be followed by generalized urticarial rash with marked swelling of the neck. The rash and swelling subsided after hospitalization and five days of treatment with oral antihistaminics and daily injections of ephedrine. Recurrent abdominal cramping without further nausea, vomiting, or diarrhea continued intermittently with the appearance of a nonspecific urethral discharge. After this acute episode, the patient complained frequently of easy fatigability, backache, headache, and mental depression. On multiple examinations the patient was found to be irritable, despondent, and complaining, although no further physical signs of illness could be detected. Roentgenological studies including gastrointestinal roentgenograms were normal. Repeated urinalyses and blood counts were normal, as were liver and kidney function studies.

Approximately six months after the onset of the acute episode, manifested by nausea,



FIG. 3. Cycloheximide being sprayed on mushroom beds located below ground in caves.

vomiting, abdominal cramping, diarrhea, and urticaria, the patient was rehospitalized after developing a leukopenia (7500 normal white blood cells depressed to 3800) with accompanying decrease in circulating granulocytes. At this time the patient was started on corticosteroids, 30 units daily, and adrenocorticotropin, 40 units every other day, with prompt decrease in symptoms and gradual disappearance of leukopenia. Bone marrow studies at this point were normal.

For more than a year the patient has remained well and active but still complains of occasional episodes of tiredness and muscle aching. Blood counts have since remained within the normal range.

DISCUSSION

The over-all observations would seem to indicate that use of streptomycin in plant disease control is not a general hazard for the agricultural worker. Skin testing revealed that workers regularly exposed to the agricultural dosage forms do not develop hypersensitivity to streptomycin more frequently than a randomly selected group.

Since it is well documented that this antibiotic has regularly elucidated untoward reactions when used in crystalline form for medical therapy, we must look to the agricultural formulations for explanation.

Streptomycin concentration in agricultural form varies from 15 to 30 per cent activity, whereas in medical usage, streptomycin is more than 95 per cent pure. Thus, the agricultural worker never encounters the pure crystalline antibiotic. It would seem therefore that daily subtle contact with concentrated streptomycin, such as a nurse receives in administering this drug to tuberculosis patients, induces a greater degree of hypersensitivity than massive contact with diluted antibiotic three to four times/year.

With reference to the contact dermatitis observed in 2 workers, two explanations are possible: first, that spray ingredients other than the streptomycin were responsible for the reaction, and second, that the skin test is not capable of predicting hypersensitivity in 100 per cent of the testees. The latter has been demonstrated with penicillin allergy determinations.⁸ Since the accessory ingredients were not tested with regard to allergenic reactions, it must be assumed that these two workers could be hypersensitive either to the so-called inert ingredients or to the antibiotic.

The problem of cycloheximide is somewhat different. This antibiotic is a poison and is so labeled by its manufacturers. Thus, while reactions to this drug used in plant disease control are rare, the potential hazard exists.

It should be pointed out, however, that the case history reported here referred to the use of cycloheximide spray in an enclosed area contrary to labeled recommendations.

SUMMARY AND CONCLUSIONS

An evaluation of the potential hazards of two antibiotics, streptomycin and cycloheximide, in plant disease control has been made. Using skin tests for hypersensitivity determinations and observations and records of untoward reactions in the field (Missouri and northern California), it was determined that field workers

spraying streptomycin did not develop hypersensitivity to this antibiotic to any greater degree than a randomly selected adult group not exposed to streptomycin.

A case of acute urticaria, nausea, vomiting, and abdominal cramping after massive topical and inhalation exposure to cycloheximide spray is reported; this acute episode was followed by a seven month period of easy fatigability, general malaise, backache, headache, mental depression, and irritability, which improved dramatically when corticosteroid therapy was instituted in an effort to correct a leukopenia that appeared some five months after cycloheximide exposure. In previous studies cycloheximide has been administered to human beings by intramuscular, intravenous, intrathecal, and intraventricular routes. No hematological abnormalities have been reported previously as a result of cycloheximide toxicity. Dosages as high as 2.7 Gm. daily have been tolerated.⁹ Gastrointestinal symptoms such as nausea, vomiting, abdominal cramping, and diarrhea and symptoms of mental depression, lethargy, and irritability have been previously reported due to cycloheximide toxicity. However, to our knowledge, acute urticaria due to cycloheximide toxicity has not been reported.

On the basis of these findings, it would seem valid to conclude that streptomycin application in plant disease control presents little hazard to the field worker. Cycloheximide, known to be toxic, has the potential ability to cause a reaction, as illustrated here, unless label recommendations warning against skin contact and inhalation are closely followed.

ACKNOWLEDGMENT

Our sincere appreciation is expressed to Dr. C. T. Shaw and Dr. W. A. Jeter of Herman, Missouri, who have allowed us to review their records of the cycloheximide case.

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The Pathogenesis of Thrombocytopenia Due to Ristocetin

EUGENE J. GANGAROSA

Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D. C.

Ristocetin, prepared from the actinomycete species *Nocardia lurida*, is an antibiotic representing two separate active antibacterial components, ristocetin A and ristocetin B. Although there is some difference of opinion,¹ the general experience has been that this antibiotic is highly efficacious in selected cases of systemic infection due to resistant staphylococci and enterococci. The toxicity of this drug involves the hematological and dermatological systems. In previous publications,²⁻⁴ the hematological complications have been summarized and the site and mechanism of action of ristocetin-induced thrombocytopenia, the most serious of the toxic reactions, have been defined.

The purpose of this paper is to summarize the laboratory studies and the Walter Reed Army Hospital clinical experience involving platelet depression and to relate these observations to the pathogenesis of ristocetin-induced thrombocytopenia. The clinical implications of these studies will be briefly discussed.

METHODS

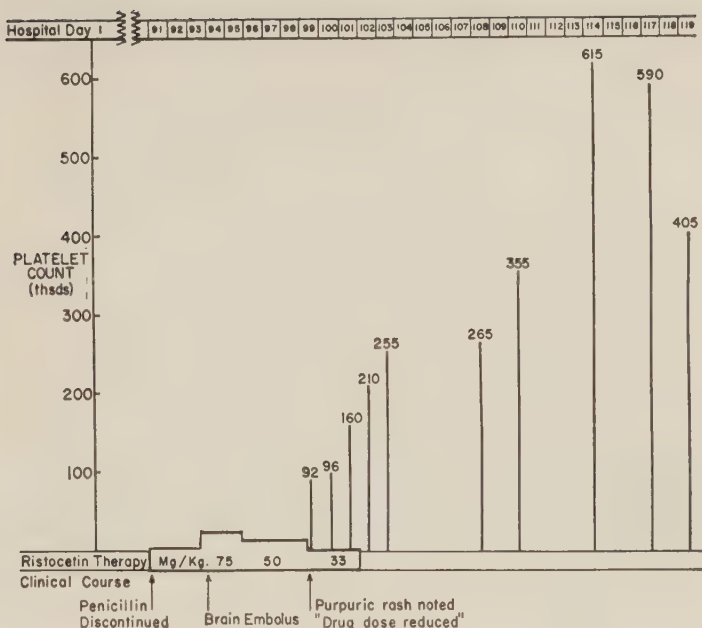
Adult albino rabbits were given varying dosages of ristocetin intravenously, using isotonic saline as the diluent in a volume not exceeding 5 ml. The drug was injected through the ear veins in one to three minutes. Blood counts were obtained before and at varying periods after injection. Platelet counts were performed by phase microscopy by the method described by Brecher et al.⁵ Animals not injected with ristocetin were studied for possible daily variation of platelet counts, and no significant variation was noted. Isotonic saline and penicillin were injected in the same manner as ristocetin and no significant adverse effects were noted. Bleeding time, clotting time, and clot retraction were evaluated in some experiments. Fibrinogen was determined by the method of Ratnoff and Menzie,⁶ fibrinolysin by the method of Adelson and Roeder.⁷

In vitro studies were performed using blood from human donors, who had not received ristocetin, collected by venipuncture with disodium ethylenediamine tetra-acetic acid as a platelet preservative and anticoagulant. The blood was incubated in test tubes at 37 C. for 30 minutes in serial dilutions of ristocetin from 0.075 mg./ml. to 10 mg./ml. The tubes were manually agitated every 10 minutes during incubation, after which platelet counts were determined. Penicillin and dihydrostreptomycin, in concentrations similar to ristocetin, had no adverse effect on platelets in these experiments.

Platelet suspensions were prepared by differential centrifugation of oxalated whole blood. They were resuspended in physiological saline in a system free of antibody and complement. These platelets were then exposed to serial dilutions of ristocetin as just described.

To a beaker containing 100 ml. of fresh human plasma from a normal donor,

FIG. 1. Platelet studies during and after ristocetin therapy in case 1.



3 ml. of ristocetin in a concentration of 5 mg./ml. was added by slowly dripping the ristocetin solution along one side of the beaker. Without further mixing, a 2 ml. specimen was obtained from the whole blood at the opposite side of the beaker and another specimen from the ristocetin-plasma mixture. A third specimen was obtained from the whole blood after thorough mixing. Paper electrophoresis was performed on these specimens using a Durrum type electrophoresis cell.

One Gm. of ristocetin in 10 ml. of normal saline was injected into the hand vein of a patient over a period of three minutes, as recommended by Terry.⁸ Platelet counts were made on blood specimens obtained from the injection site prior to ristocetin injection, from a proximal site in the same vein during injection, and from a vein of the opposite arm three minutes after injection.

RESULTS

Case Reports. Platelet depression occurred in the following cases. Ristocetin was administered intravenously with either glucose or normal saline as the diluent, as recommended by the manufacturer.

Case 1. A 43 year old man completed an 11 week course of combined streptomycin and penicillin for enterococcal endocarditis on the ninety-first hospital day. Three days later a temperature of 102 F., tachycardia, and a left-sided homonymous hemianopsia developed. Numerous blood cultures were sterile, but because of the possibility of active endocarditis, ristocetin was started. Secobarbital was the only other medication given. The patient was given 4.5 Gm. of ristocetin (75 mg./Kg. of body weight) on the ninety-fourth day and 3 Gm. (50 mg./Kg.) from the ninety-fifth to the ninety-ninth hospital days. On the ninety-ninth day, fever and a purpuric rash were noted, a Rumpel-Leede test was "strongly positive," and platelet count (fig. 1) was 92,000 (a peripheral smear on admission had indicated an adequate number of platelets). The dosage of ristocetin was decreased to 2 Gm. daily (33 mg./Kg. of body weight), and the platelet count returned to normal. There was no change in the other blood elements.

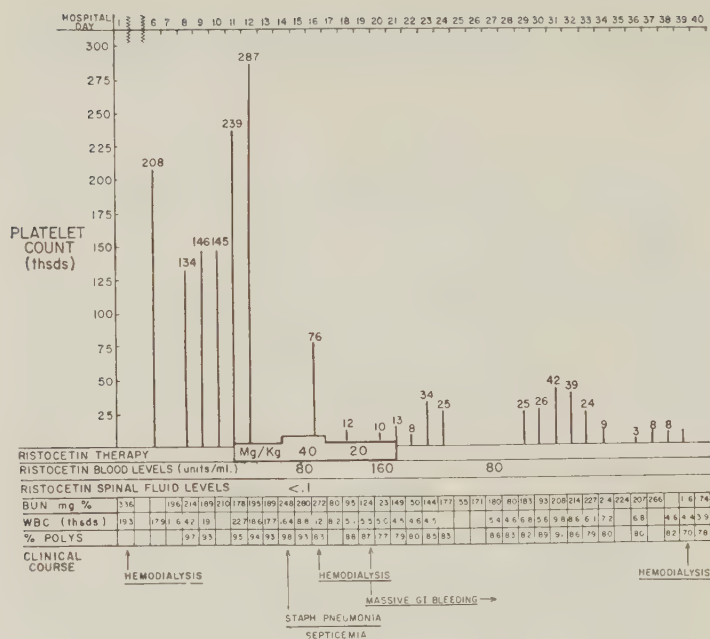


FIG. 2. Platelet studies before, during, and after ristocetin therapy in case 2.

Case 2. A 36 year old man was hospitalized because of acute renal insufficiency after trauma. On the sixth hospital day fever developed, and blood and sputum cultures grew hemolytic *Staphylococcus aureus*. The patient was treated with novobiocin and oleandomycin and improved initially, but on the fourteenth hospital day he again became febrile. Physical examination and roentgenographic study revealed lobar pneumonia on the right side. All previous antibiotics were discontinued, and ristocetin, 3 Gm. daily (40 mg./Kg. of body weight), and hydrocortisone were started. A prompt and dramatic clinical response was noted, with resolution of the roentgenographic findings. On the third day of ristocetin therapy, the dosage was reduced to 1.5 Gm. daily (20 mg./Kg. of body weight) for another four days. During this time the patient was uremic, and the urinary output ranged between 400 and 900 ml./day. On the fourth and fifth days of therapy melena was noted, and the hematocrit had fallen from 32 to 19 per cent. The platelet count at that time was noted to be markedly depressed (fig. 2), and the white cell count fell to 4500 from levels of 12,600 before ristocetin treatment. Platelet depression continued even after ristocetin was stopped, but the blood level seven days later was markedly elevated. Severe bleeding, which required frequent small transfusions, persisted and contributed to the patient's ultimate death on the forty-first hospital day.

At postmortem examination the bone marrow revealed a normal number of megakaryocytes and other cellular elements.

Case 3. A 50 year old man was hospitalized because of acute renal insufficiency after acute pancreatitis with protracted hypotension. On the tenth hospital day fever and chills developed. Sputum, throat, and stool cultures grew *Staph. aureus*. The patient was treated with chloramphenicol for 48 hours without improvement. He was then given ristocetin, 2.25 Gm. daily (30 mg./Kg. of body weight), on the twelfth hospital day as the only medication, with marked improvement. After two days the dosage was reduced to 1 Gm. daily (20 mg./Kg.). During this therapy severe gastrointestinal bleeding was noted concomitant with severe platelet depression (fig. 3). After seven days of therapy, ristocetin was discontinued; the platelet count returned to normal, and bleeding ceased. On the thirty-second hospital day the patient again became febrile, and *Staph. aureus* was grown from a blood culture. Ristocetin, 3 Gm. daily (40 mg./Kg. of body weight), was given in addition to chloramphenicol, streptomycin, and sulfisoxazole. Platelet depression again ensued while azotemia improved and the infection

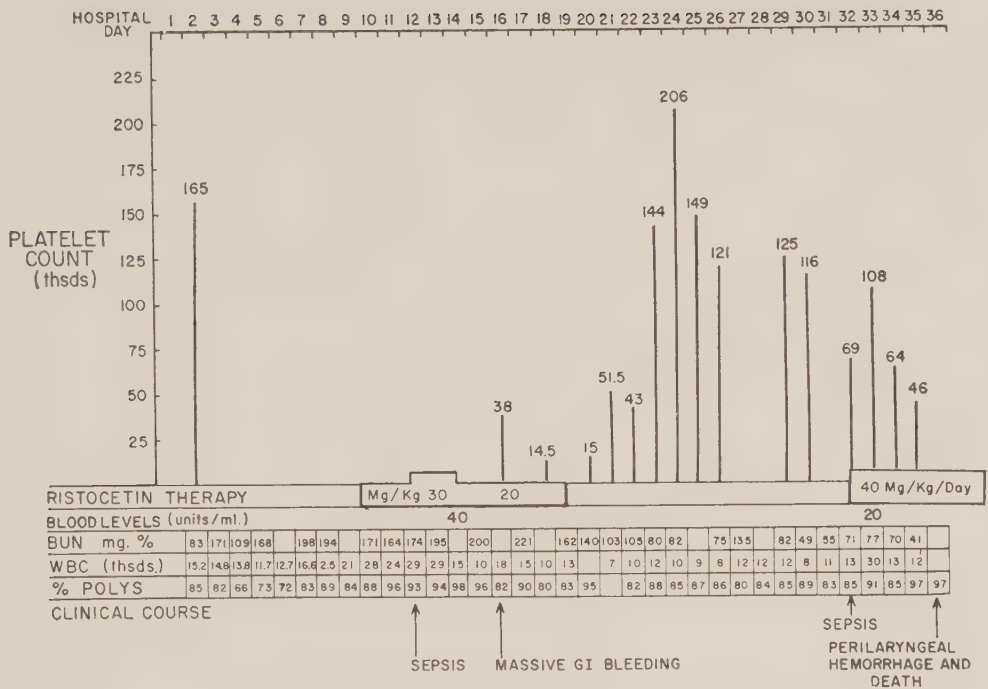


FIG. 3. Blood studies before, during, and after ristocetin therapy in case 3.

was being eradicated. A thromboelastogram was abnormal, indicating poor platelet function. On the thirty-fifth hospital day the platelet count fell to 46,000; the patient had a perilaryngeal hemorrhage and died. On postmortem examination the bone marrow was normal and contained normal numbers of megakaryocytes.

Case 4. The patient was a 37 year old Negro man in whom staphylococcal pneumonia developed after an automobile accident. The infection progressed radiographically and clinically in spite of antibiotic treatment with kanamycin, erythromycin, and chloramphenicol. A tracheostomy was performed to control secretions. The patient was given a loading dose of ristocetin, 25 mg./Kg. of body weight, followed by a maintenance dosage of 35 mg./Kg. of body weight per day. He was maintained on erythromycin and chloramphenicol. There was a dramatic clinical response with gradual resolution of the pulmonary lesions. The platelet response to ristocetin is depicted in figure 4. He had no evidence of impaired renal function. The bone marrow on the last day of ristocetin treatment revealed a mild increase in megakaryocytes with normal platelet formation. An intracutaneous injection of 5 mg. of ristocetin and a patch containing 50 mg. of ristocetin applied to the skin produced no reaction in three days. The tourniquet test, bleeding and clotting times, and clot retraction were normal throughout, and there was no clinical evidence of bleeding. The thromboelastogram and the prothrombin consumption test were abnormal and compatible with thrombocytopenia. Transient neutropenia and eosinophilia were noted after ristocetin therapy was discontinued. Four weeks after full recovery, the patient was challenged with 500 mg. of intravenous ristocetin without evidence of platelet depression.

Case 5. This patient was a 61 year old white man in whom staphylococcal peritonitis and pneumonia developed after an elective abdominal wall herniorrhaphy. He was started on ristocetin, 40 mg./Kg. of body weight, as a loading dose with an initial maintenance dosage of 60 mg./Kg. of body weight per day for two days and 20 to 40 mg./Kg. of body weight per day thereafter, a currently recommended therapeutic regimen. He was also maintained on chloramphenicol. The patient had no evidence of renal impairment to account for excessive ristocetin accumulation. The platelet response to ristocetin is depicted in figure 5. Two tran-

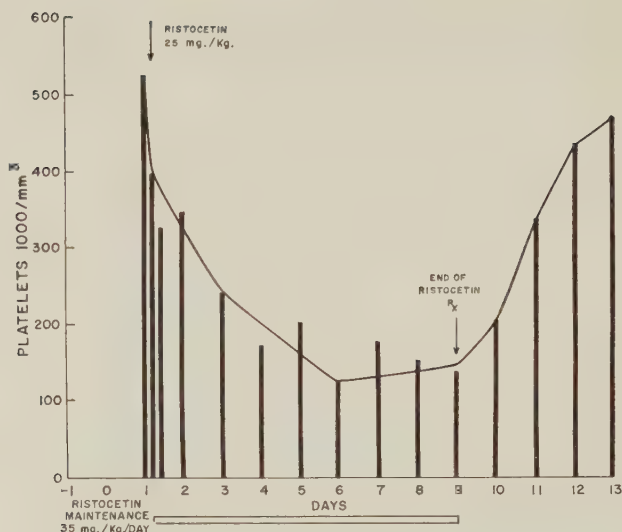


FIG. 4. Platelet studies before, during, and after ristocetin therapy in case 4.

sient episodes of thrombocytopenia occurred. The platelet count returned to normal each time after a reduction in the maintenance dosage of ristocetin. The second episode of thrombocytopenia was associated with melena and skin ecchymosis. There was a drop in hemoglobin from 11 Gm./100 ml. to 8.7 Gm./100 ml., for which 2 units of blood was administered. The infection was successfully eradicated.

Human Studies. Four weeks after full recovery, patient 4 was challenged with 500 mg. of intravenous ristocetin without evidence of platelet depression or other indications of toxicity. In another patient, with a normal platelet count of 350,000/cu. mm., the administration of 1 Gm. of ristocetin in 10 ml. of saline in a hand vein resulted in a complete lysis of platelets in the blood specimen obtained from a proximal site during the injection.

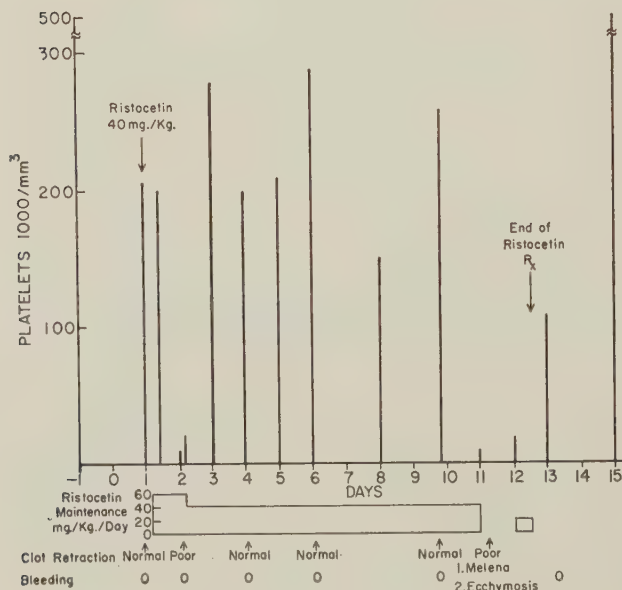


FIG. 5. Coagulation studies before, during, and after ristocetin therapy in case 5.

TABLE I

Platelet Response of Rabbits to Ristocetin Administered Daily

Animal	Day of ristocetin schedule, mg./Kg.				Days for maximum platelet depression	Platelet counts, 1000/cu. mm.		Platelet destruction, per cent of control count
	1	2	3	4		Preinjection control	Post injection	
1	40	80	200	200	4	1877	715	62
2	40	80	200		3	490	161	67
3	70	140			2	384	34	91
4	200	275			2	837	250	70
5	70	140			2	690	284	59
6	50	130			2	600	90	85

Animal Studies. Table I shows the results of daily platelet studies in rabbits given ristocetin intravenously in dosages of 40 to 275 mg./Kg. of body weight per day. Significant platelet depression was noted in 6 of 7 animals studied. None of these animals developed anemia or leukopenia.

Table II depicts acute thrombocytopenia after the intravenous administration of ristocetin in dosages between 50 and 200 mg./Kg. of body weight. The time required for maximum platelet depression varied from five minutes to five hours. The detailed platelet changes in one experiment are depicted in figure 6. Blood obtained prior to ristocetin injection clotted normally and there was good clot retraction. The bleeding time was also normal. After receiving a total of 550 mg./Kg. of body weight in four separate injections over a four hour period, the animal had a bleeding time of 30 minutes and the blood did not clot after five days. After the fourth injection of ristocetin, the animal developed fatal toxic convulsions. The serum level of ristocetin was 1200 µg./ml. and the platelet count was 23,000/cu. mm.

Fibrinogen levels were determined in 3 rabbits before and after ristocetin injection. There was a consistent fall in the circulating fibrinogen in each of the experimental animals, with the maximum fibrinogenemia at two hours (table III). In the 1 rabbit tested, there was evidence of marked fibrinolysis.

Megakaryocytes were abundant and platelet production was normal in the bone marrow of animals with severe thrombocytopenia. Examination of other organs revealed no abnormalities.

TABLE II

Acute Platelet Response of Rabbits to Ristocetin

Animal	Dosage of ristocetin, mg./Kg.	Time for maximum depression of platelet	Platelet counts, 1000/cu. mm.		Platelet destruction, per cent of control count
			Preinjection control	Post injection	
1	200	2 hours	436	235	46
2	100	5 minutes	700	392	44
3	50, 200, 150, 150*	5 hours	735	23	97

* See figure 3.

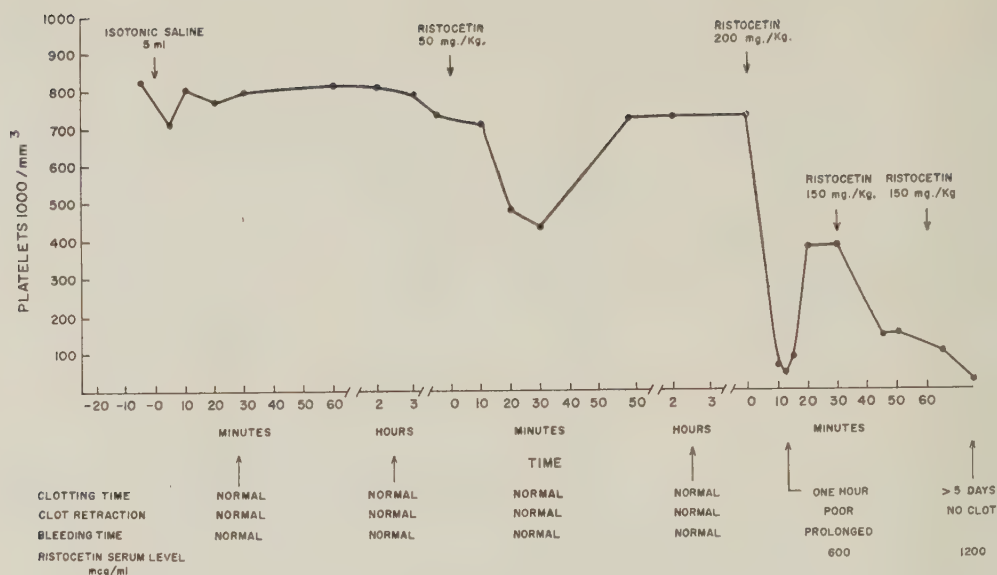


FIG. 6. Acute platelet response of a rabbit receiving ristocetin.

In Vitro Studies. The *in vitro* effects of ristocetin on platelets from human donors are summarized in figure 7 and table IV. Platelet lysis occurred in concentrations exceeding 310 $\mu\text{g./ml.}$ but was not always directly proportional to the concentration of drug. Platelet stroma could be identified microscopically in specimens in which lysis had occurred. Agglutination of platelets occurred in concentrations of ristocetin between 0.15 mg./ml. and 1 mg./ml.

Washed platelets suspended in saline were lysed when exposed to ristocetin in concentrations exceeding 0.5 mg./ml. The extent of platelet lysis in these experiments was directly proportional to the concentration of ristocetin.

The effects of ristocetin on human platelets and fibrinogen in the beaker experi-

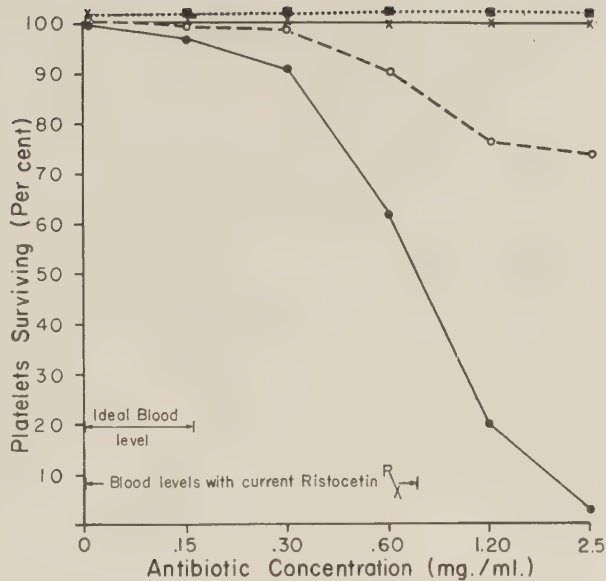
TABLE III

Fibrinogen Levels in Rabbits After Intravenous Ristocetin

Time after ristocetin	Fibrinogen, mg./100 ml. (average of 2 determinations)		
	Animal 1, 100 mg./Kg.	Animal 2, 100 mg./Kg.	Animal 3, 125 mg./Kg.
Control	500	450	560
5 minutes	455 (230*)	410	—
15 minutes	415 (250*)	—	—
30 minutes	345 (270*)	450	260
60 minutes	320 (220*)	450	240
120 minutes	250 (200*)	125	350
24 hours	540 (540*)	470	580
Maximum decrease in fibrinogen	50%	72%	67%

* After 24 hours' incubation of the clot, the further decrease in fibrinogen indicates the presence of fibrinolysin.

FIG. 7. Lysis of human platelets incubated in vitro with ristocetin. ■ · · · ■, penicillin (average, 3 experiments); × — ×, dihydrostreptomycin (average, 3 experiments); o — — o, tetracycline (average, 4 experiments); ● — — ●, ristocetin (average, 16 experiments).



ment are depicted in figure 8. Rouleau formation and platelet-fibrin clots, which occur when ristocetin in a concentration exceeding 2.5 mg./ml. is added to freshly drawn human blood in a silicone syringe, are depicted in figure 9. A thromboelastogram shown in figure 10 reflects the delayed clot formation and the poor quality

TABLE IV

The Response of Platelets from Human Donors to Ristocetin in Vitro

Donor	Ristocetin concentration, mg./ml.		Platelet counts, 1000/cu. ml.		% platelet lysis
	Lysis occurred between	Maximum lysis at	Without ristocetin	With maximum lysis	
1. Normal	0.63- 5	1.25	195	0	100
2. Normal	0.63-10	0.63	340	0	100
3. Normal	0.63- 5	2.5	237	6	97
4. Normal	1.25- 5	2.5	239	0	100
5. Idiopathic thrombo- cytopenia	1.25-10	5.0	95	0	100
6. Normal	1.25- 5	2.5	234	0	100
7. Normal	0.63-10	2.5	229	7	97
8. Normal	1.25-10	2.5	335	30	90
9. Normal	0.63-10	1.25	385	0	100
10. Sick cell trait	1.25-10	5.0	371	11	97
11. Chronic myeloge- nous leukemia	0.63-10	2.5	1320	135	90
12. Normal	0.63-10	0.63	142	0	100
13. Normal	1.25-10	2.5	181	0	100
14. Normal	1.25- 5	2.5	181	3	98
15. Iron-deficiency anemia	0.31-10	2.5	350	4	99
16. Iron-deficiency anemia	0.31-10	2.5	350	1	99
17. Normal	1.25- 5	2.5	184	18	90
18. Hypogamma- globulinemia	1.25- 5	2.5	195	0	100

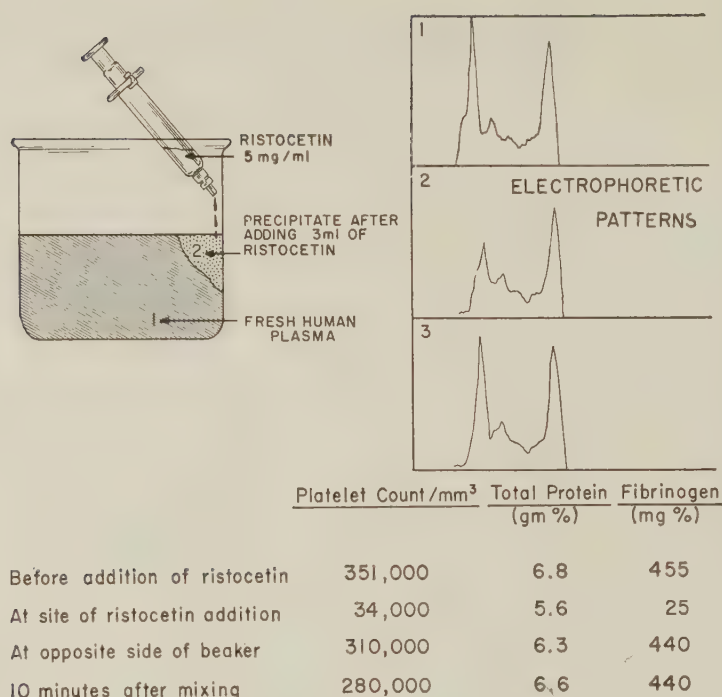


FIG. 8. The Beaker Experiment. The electrophoretic patterns numbered 1, 2, and 3 represent respectively specimens of human plasma: (1) before the addition of ristocetin, (2) at the site of ristocetin addition, and (3) after thorough mixing. The first peak in the electrophoretic pattern represents fibrinogen. The decrease in the fibrinogen peak in pattern number 2 reflects the denaturation of fibrinogen by ristocetin. This is confirmed by the quantitative determination of fibrinogen by another method.

of the clot when ristocetin in the same concentration is added to freshly drawn blood in a siliconized syringe.

DISCUSSION

The drug-induced thrombocytopenias may be classified on the basis of the site and mechanism of toxic action as follows: thrombocytopenia involving megakaryocytic (bone marrow) depression: due to an immune mechanism (probably chloramphenicol⁹) and due to a direct toxic effect (antimetabolites such as 6-mercaptopurine); thrombocytopenia involving circulating platelets: due to an immune mechanism (quinidine,¹⁰ sedormid,¹¹ novobiocin¹²) and due to a direct toxic effect (ristocetin).

This discussion will be limited to ristocetin thrombocytopenia.

Platelet depression is not a rare complication of ristocetin therapy. Six cases have been reported by this author and 9 cases have been noted by others.¹³⁻¹⁷ The present animal and in vitro studies confirm that ristocetin is capable of producing thrombocytopenia.

The site of action of ristocetin-platelet destruction is the circulating blood. Megakaryocytes are present in normal numbers, and there is active platelet production in the bone marrow of patients and of rabbits with ristocetin-induced thrombocytopenia. Additional evidence of peripheral platelet depression is obtained from the platelet lysis, which occurs in vitro when ristocetin is incubated with either normal human blood or platelet suspensions. Furthermore, the rapid disappearance of platelets after large doses of ristocetin are injected into rabbits points to a peripheral, rather than a central, effect.

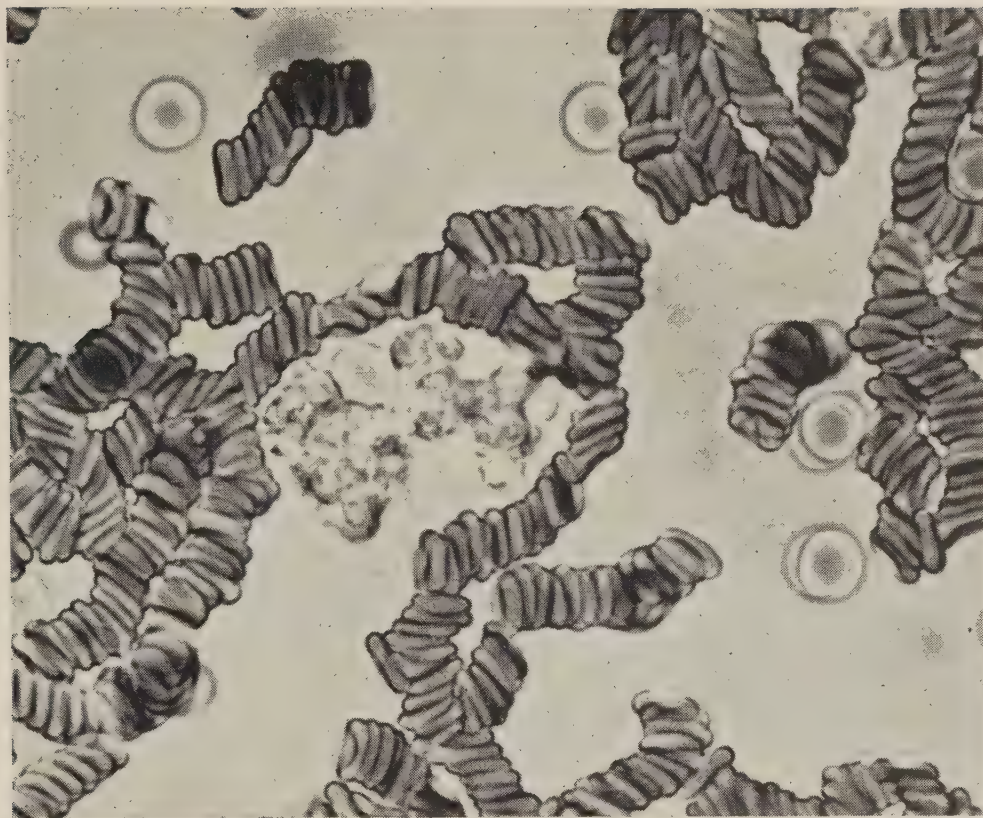


FIG. 9. Note the large platelet-fibrin clot in the center, and the rouleau formation which occurs when freshly drawn human blood is added to ristocetin in a concentration exceeding 2.5 mg./ml.

An immune mechanism cannot be invoked to explain ristocetin thrombocytopenia. The evidence for a direct toxic effect and against an immune mechanism is as follows: Platelet lysis occurs *in vitro* in a system containing only ristocetin, platelets (from a normal donor never exposed to ristocetin), and physiological saline. Complement and antibody are therefore not necessary for the reaction. Also, there is no platelet depression in a patient who has recovered from ristocetin-induced thrombocytopenia when challenged with a single 500 mg. dose of the drug one month after the initial exposure. The immunothrombocytopenias have been characterized by a marked platelet depression when so challenged by the offending drug.^{10, 12, 18, 19} Also, immunoreactions are usually associated with prior exposure to the offending drug. In contrast, thrombocytopenia can be demonstrated in rabbits within 30 minutes after the initial exposure to ristocetin. Finally, platelet depression is easily reversed in animals and man merely by decreasing the dosage of the drug. This dose relationship implies direct toxicity, rather than an immunoreaction.

The characteristic drop in platelets and fibrinogen in rabbits followed by the development of fibrinolysis suggests the possibility of an incomplete intravascular reaction. This is analogous to the hemoclastic reaction that may follow plasma transfusion.²⁰ The probable explanation for such a reaction is that platelets, injured

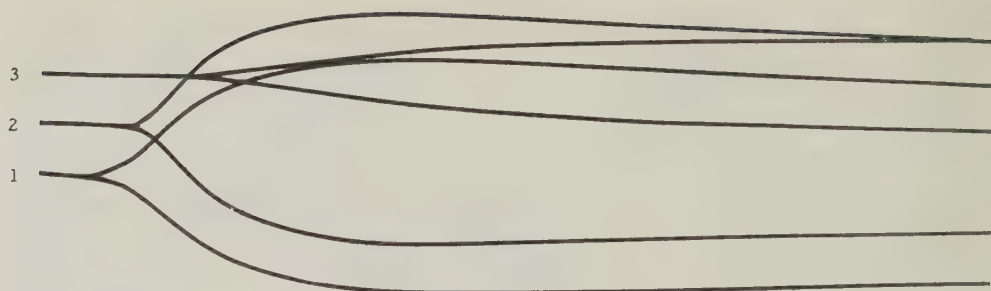


FIG. 10. Depicted above are three thromboelastographs. A thromboelastograph is a photographic representation of the clotting process.²⁶ As the clot forms, the straight line separates into two branches. The firmer the clot, the wider the branches. Note that the pattern representing the clotting sequence with ristocetin and whole blood is different from the controls in that the initiation of clot formation is delayed and the clot which forms is less rigid. 1, Whole blood control; 2, whole blood plus 2.5 mg./ml. penicillin; 3, whole blood plus 2.5 mg./ml. ristocetin.

by high concentrations of ristocetin, agglutinate and disintegrate, releasing thromboplastin. Thrombin and finally fibrin are formed in minute amounts. The hemostatic balance is upset and a fibrinolytic response is invoked to prevent irreparable damage to the organism. The dose-response relationship (fig. 7) suggests that the local insult of concentrated solutions of ristocetin at the site of intravenous administration may play an important role in initiating platelet destruction. This phenomenon of local platelet destruction should be considered in evaluating the recommendation of one author that ristocetin be used in a concentration of 100 mg./ml.⁸

Further evidence of the local toxic effects of high concentrations of ristocetin is provided by the "beaker" experiment (fig. 9) and the absence of platelets in the blood specimen obtained from the proximal arm vein of a patient during the injection of ristocetin. These data explain the very transient episodes of thrombocytopenia noted by us and others^{13, 14, 16, 17} and the ease with which this type of platelet depression may be quickly reversed merely by decreasing the dosage of the drug or discontinuing it. Thrombocytopenia may be the end result of repeated episodes of lysis of platelets at the site of the administration of concentrated solutions of ristocetin. This local platelet destruction occurs at a rate faster than they can be produced by the bone marrow megakaryocytes, resulting in a deficit of circulating platelets in some persons.

Since the introduction of ristocetin in clinical medicine, there has been a significant change in this drug, which can be appreciated by comparing the deep brown color of the original ristocetin mixture with the nearly white material that is currently marketed. This has been attributed by the manufacturer to a reduction in impurities and a change in the ristocetin A and ristocetin B ratio. What effect these changes will have on the future incidence of toxic reactions cannot be predicted at this time. Until the toxicity of the purified material is defined, it is recommended that patients receiving ristocetin be carefully observed for platelet toxicity either by phase platelet counts or by frequent evaluation of the platelets in the peripheral smear.

Leukopenia,^{3, 13, 21} an infrequent complication of ristocetin therapy, may occur days or even weeks after the drug has been discontinued, and it is frequently accompanied by eosinophilia.¹³ The appearance of this complication after the drug

has been discontinued and its frequent association with eosinophilia suggest an allergic basis for this reaction.

Rouleau formation and hemolysis can be demonstrated when erythrocytes are exposed to concentrations of ristocetin that cause platelet agglutination and lysis. The relationship between these observations and the anemia sometimes seen in patients on ristocetin therapy^{3,22} is unknown. The most likely cause of this complication is unrecognized thrombocytopenia with disguised bleeding, rather than massive hemolysis.

The clinical significance of the protein denaturation that occurs when ristocetin in concentrations exceeding 0.25 mg./ml. is added to plasma, bovine albumin, or fibrinogen solutions is unknown.

The exact physical alterations of the platelet surface resulting from the action of ristocetin remain to be elucidated. Perhaps the reason there has been no spontaneous emergence of ristocetin-resistant staphylococci is that the drug does not act biochemically by blocking metabolic pathways, but physicochemically by altering the proteins of the bacterial cell wall.

The antistaphylococcal potency of ristocetin has been effectively demonstrated by several investigators²¹⁻²⁵ and has been confirmed by us. The adverse reactions accompanying the use of this drug are for the most part due to the use of excessive dosages and possibly high concentrations. It is the author's belief that dosages greater than 50 mg./Kg. of body weight per day and solutions more concentrated than 2 mg./ml. should not be used. The drug should be reserved for resistant staphylococcal and enterococcal infections exclusively.

SUMMARY

An experimental approach to the mechanism of ristocetin-induced thrombocytopenia has been presented. This complication of ristocetin therapy is the result of a direct toxic effect of the drug on circulating platelets and may occur even with currently recommended therapeutic regimens. It is independent of humoral factors and is related to the dosage of the drug. Evidence is presented that suggests that thrombocytopenia may be the end result of repeated episodes of lysis of platelets at the site of the administration of concentrated solutions of ristocetin.

ACKNOWLEDGMENTS

The author is particularly indebted to Lt. Col. Frank Miller, Col. William H. Crosby, and Dr. Geoffrey Edsall for their assistance and guidance. Ristocetin blood levels were kindly performed by Dr. Monroe Romansky and the Abbott Laboratories.

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WILEY H. HARRISON

Chicago, Ill.

In recent years, there have been many papers presented in various medical journals indicating that deafness occurs frequently due to dihydrostreptomycin therapy. Most of this early work was done in institutional programs with controlled studies in which dosage of the drug used was moderate in amount.¹ There certainly can be no quarrel with the use of a drug as a last resort, lifesaving procedure, taking into account the calculated risk of the toxic effect.

However, we are continuing to see in practice irreversible hearing loss attributed to relatively small amounts of dihydrostreptomycin used in combination with penicillin prophylactically and in minor infections. A series of these cases was reported in conjunction with other otolaryngologists recently.²

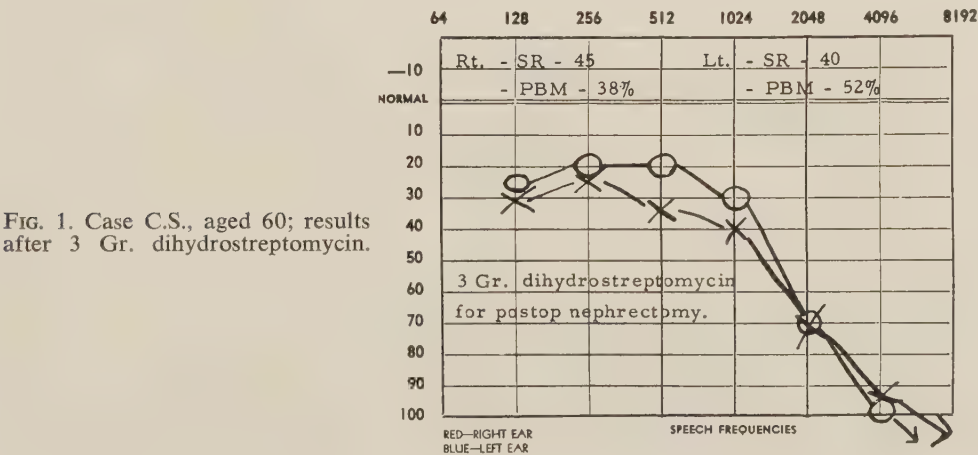
Even in recent months we have seen an alarming number of patients with hearing impairment after dihydrostreptomycin therapy. The illustrations show a series of audiograms with a brief case history revealing hearing loss after dihydrostreptomycin therapy.

As can be seen in the illustrations, many cases of moderate or mild loss in pure tone from the use of this drug are accompanied by a disproportionate impairment of speech discrimination.

It has been difficult to obtain evidence from colleagues to the effect that dihydrostreptomycin is superior to streptomycin for reasons other than that the vertigo caused by the latter is annoying to the patients.³

Dihydrostreptomycin is particularly treacherous because of the small dosage apparently necessary to produce hearing loss and because of the latent period between the administration of the drug and the onset of the hearing loss.

There is no known effective treatment for this loss. It has been reported that



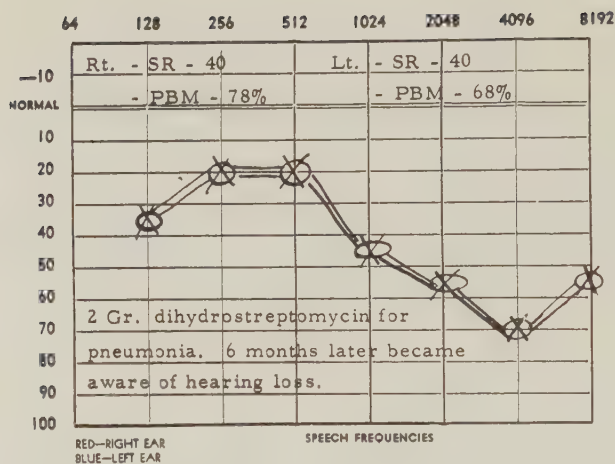


FIG. 2. Case W.B., aged 46; results after 2 Gr. dihydrostreptomycin.

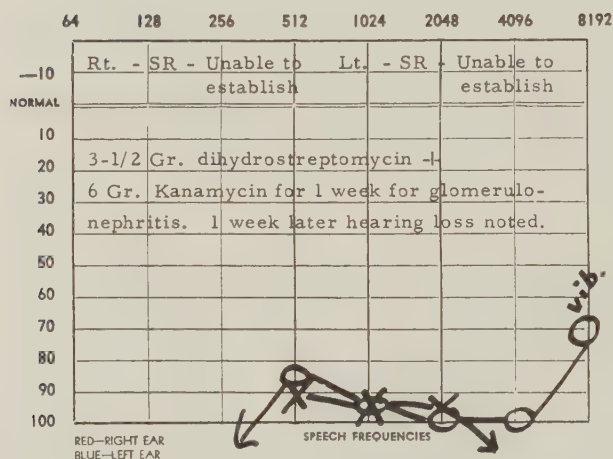


FIG. 3. Case D.R., aged 28; results after 3.5 Gm. dihydrostreptomycin and 6 Gm. kanamycin.

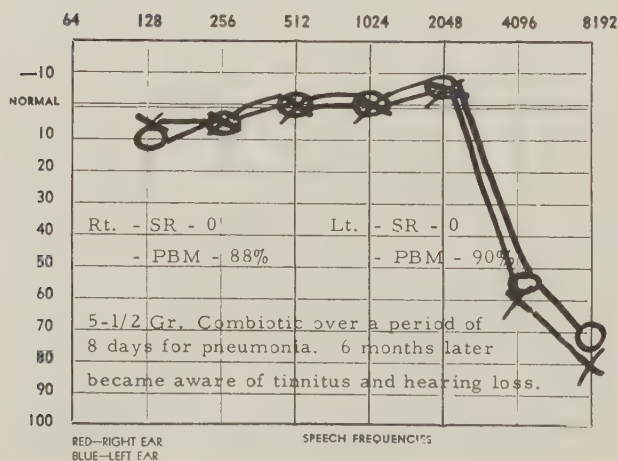


FIG. 4. Case J.W., aged 11; results of 5.5 Gm. of penicillin G and dihydrostreptomycin base.

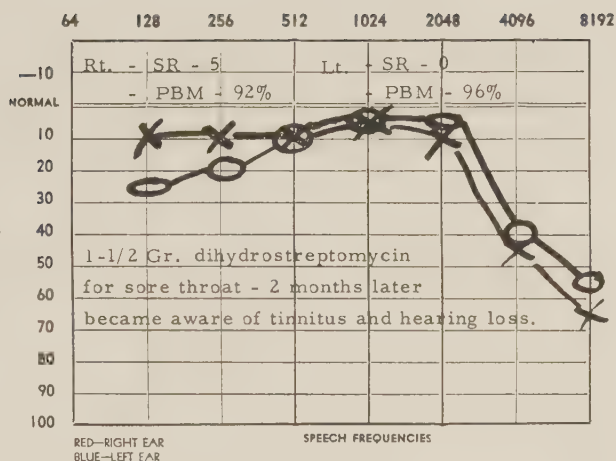


FIG. 5. Case I.B., aged 36; results of 1.5 Gm. dihydrostreptomycin.

large amounts of vitamin B₁ used parenterally are of some value. In practice I have been unable to substantiate this.

Dihydrostreptomycin is at present used in many commercial preparations with penicillin in which its presence is not clearly indicated, and in cases in which its use cannot be justified.⁴

It is recommended that dihydrostreptomycin be omitted from commercial combinations of antibiotics.

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The Effect of Dihydrodesoxystreptomycin on the Function of the Eighth Nerve

EARL W. CHRISTENSEN, WALLACE E. HERRELL, AND JAMES T. GILBOY, JR.

Lexington Clinic and Julius Marks Sanatorium, Lexington, Ky.

The ototoxicity of streptomycin and dihydrostreptomycin is well recognized and well documented. The shift from an almost specific vestibular toxicity for streptomycin to an almost specific cochlear toxicity for dihydrostreptomycin is a striking example of the modification of antibiotic activity that results from modification of its structure. The incidence of toxicity has been a deterrent to the prolonged use of streptomycin and dihydrostreptomycin.

Dihydrodesoxystreptomycin sulfate* is a chemical analogue of streptomycin developed by Ikeda and his associates^{1,2} at the Scientific Research Institute in Tokyo. Their studies suggested that this preparation was as active as or slightly more active than the parent compound. One of us³ reported on preliminary studies using dihydrodesoxystreptomycin for the short-term therapy of patients suffering from a variety of acute infections. In addition, 25 patients who had culturally proved pulmonary tuberculosis were treated with dihydrodesoxystreptomycin, 1 Gm. twice weekly. In these preliminary studies there was no clinical evidence of neurotoxicity.

The present study was designed to test the possible neurotoxicity of dihydrodesoxystreptomycin; it was begun April 23, 1959. Nine patients who were under observation and treatment for pulmonary tuberculosis at Julius Marks Sanatorium were selected for this study. The group included 6 men and 3 women. The ages of the patients ranged from 20 to 77 years (table I).

TESTS EMPLOYED

Pretreatment audiograms, postural tests for vertigo, caloric stimulation of the vestibular labyrinth, and the Rhomberg test were done on all 9 patients. All of these tests were repeated at monthly intervals after treatment was started and one and two months after treatment was completed. Before treatment was started, preliminary examination of the ears, nose, and throat was essentially normal in all cases. Treatment consisted of the daily administration of 1 Gm. of dihydrodesoxystreptomycin by the intramuscular route. Treatment was continued for 90 days (three months) in 8 cases. In the remaining case treatment was discontinued after 60 days, not because of toxicity but because the diagnosis of tuberculosis was not confirmed by culture.

Standard audiograms were performed in the usual manner. The Rhomberg test was performed with the patient's head upright, inclined forward, backward, and over each shoulder. The postural test was done with the patient in the supine position, with the head turned right and then left. The patient then rose quickly

* The dihydrodesoxystreptomycin used in these studies was kindly supplied by Eli Lilly & Co., Indianapolis, Indiana.

TABLE I
Dihydrodesoxystreptomycin: 9 Cases

Patient	Age, yr.	Sex	Race	Number of days treated
1	77	M	White	90
2	44	M	Negro	90
3	24	F	Negro	90
4	58	M	White	90
5	69	M	White	60*
6	34	M	Negro	90
7	20	F	Negro	90
8	45	F	Negro	90
9	53	M	Negro	90

* Cultures did not confirm diagnosis of tuberculosis. Treatment was discontinued.

from the supine to the sitting position and finally was studied with the head hanging and then returned to the upright position. The caloric test was done using 2 ml. of ice water, which was instilled under direct vision and removed after 20 seconds. Nystagmus was observed for duration, amplitude, and rapidity, with the patient supine, with the head on a pillow, and looking straight forward. The right ear was always tested first, since the left ear was tested without a rest period. At no time from the beginning of these studies did the examiner consult or have available the results of the tests done previously.

RESULTS

The results of the vestibular studies performed on these patients are shown in table II. The postural test remained normal in all 9 cases before, during, and after treatment. The Romberg test was positive before treatment was started in case 4, but was normal thereafter. The Romberg test was normal in case 5 (69 year old patient) before treatment, positive after 30 and 60 days, and normal again at four and one half months. In case 9 the Romberg test was recorded as slightly positive at 30 and 60 days, but this was thought to be due to nothing more

TABLE II
Vestibular Studies

Case	Pretreatment			During treatment			Post-treatment		
	Postural	Rhomberg	Caloric	Postural	Rhomberg	Caloric	Postural	Rhomberg	Caloric
1	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	Normal	Positive	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	Normal	Normal	Normal	Normal	Positive	Normal	Normal	Normal	Normal
6	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	Normal	Normal	Normal	Normal	Slightly positive	Normal	Normal	Normal	Normal

TABLE III

Audiograms

Case	Pretreatment*	During treatment	Post-treatment	
			1 month	2 months
1	Normal	Normal	Normal	Normal
2	Normal	Normal	Normal	10 decibel loss at 8000 cycles (right ear)†
3	Normal	Normal	Normal	Normal
4	Normal	Normal	30 decibel loss at 8000 cycles (bilateral)†	50 decibel loss at 8000 cycles (bilateral)†
5	Normal	Normal	Normal	Normal
6	Normal	Normal	Normal	Normal
7	Normal	Normal	Normal	Normal
8	Normal	Normal	Normal	Normal
9	Normal	Normal	Normal	Normal

* Pretreatment audiogram regarded as normal for this study.

† As compared with pretreatment audiogram.

than moderate unsteadiness. It is our opinion that these occurrences do not represent evidence of toxicity that could be attributed to the dihydrodesoxystreptomycin. The caloric tests were normal during the pretreatment, treatment, and post-treatment periods.

The pretreatment audiograms in all 9 cases did not influence selection of cases. The variations from normal were compatible with age and formed the individual comparison base line. During the 90 day period of treatment there was no evidence of impairment of auditory function. At the end of the fifth month (two months after treatment was discontinued), a 10 decibel loss of hearing was found in the right ear at 8000 cycles in case 2. This person's hearing had remained stable in all previous tests. In case 4, one month after treatment was discontinued, a hearing loss of 30 decibels, which was bilateral, was found at 8000 cycles. Two months after treatment was discontinued, the bilateral hearing loss was 50 decibels at 8000 cycles. The results of these studies are shown in table III.

Paresthesias, which are not infrequently seen after the administration of streptomycin, were not observed in any of this group of 9 patients. When compared with other forms of streptomycin, dihydrodesoxystreptomycin caused less pain at the site of injection.

SUMMARY

This study was designed to test the possible toxic effect of dihydrodesoxystreptomycin on the function of the eighth nerve. Nine patients who were under observation and treatment for pulmonary tuberculosis were studied. Eight of these patients received 1 Gm. of dihydrodesoxystreptomycin by the intramuscular route daily for 90 days. In the remaining case administration of dihydrodesoxystreptomycin was discontinued after 60 days, not because of toxicity but because the diagnosis of tuberculosis was not confirmed by culture. Pretreatment studies of the function of

the eighth nerve were used as the comparison base line. Vestibular studies and audiograms were done at monthly intervals during the three month period of treatment and the two month period after treatment. The postural and caloric tests remained normal during and for two months after treatment in all 9 cases. In all cases the Rhomberg test was normal two months after treatment had been discontinued.

The administration of 1 Gm. of dihydrodesoxystreptomycin daily for 90 days did not appear to cause any impairment of vestibular function. In 1 of the 9 patients included in this study, a 30 decibel bilateral hearing loss at 8000 cycles was first noted one month after treatment with dihydrodesoxystreptomycin had been completed. Two months after treatment had been discontinued this patient's hearing loss bilaterally was 50 decibels at 8000 cycles. In another case a significant hearing loss in the right ear appeared two months after treatment had been discontinued. These studies lead us to conclude that dihydrodesoxystreptomycin is not free of toxic effect on the auditory branch of the eighth nerve.

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Automation in Turbidimetric Assays

DOROTHY M. HUCKE AND CHARLES H. ROCHE

Biological Control Laboratories, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

Two years ago we investigated the possibility of adapting high speed data processing techniques to routine microbiological assays. A high speed electronic computer was already available for assay computations; but a computer works with cards. It would not be economical to produce the cards by manual punching from a laboratory notebook. To develop a system that would be financially attractive some method had to be found to produce automatically the cards right in the laboratory.

A committee of two, one trained in biostatistics, the other in data processing, was assigned to this problem. We made a survey of the manufacturers of electronic equipment and found only one, the International Business Machines Corporation, had a complete data recording system that satisfied our needs. This system, termed by its manufacturer, automatic production recording, was offered with various components from which we selected those which fulfilled our requirements.

The changes that this system has produced in our laboratory can only be described as revolutionary. Today we can do more work—and better work—with fewer people; we have largely rid ourselves of the fear of human error and we have a deeper sense of confidence in our test results; we have extricated our supervisory people from under a mountain of paper work and freed them to put their time and skills to better use.

The reorganization of our laboratory operation to fit the automatic production recording system is shown in figure 1. All samples enter the laboratory through the receiving station. For every sample, we generate a weight-dilution master card containing the necessary sample information. This card is sent to a keypunch operator who punches the information that is handwritten on the card. In the meantime, the sample is processed through the laboratory and reaches the reading station where light transmissions are recorded. Here another card containing the turbidity readings is automatically produced. Both the weight-dilution card and the reading card are sent to the IBM 650 electronic computer, which produces an output card containing all of the original sample information as well as the computed potency. The output cards are then used to produce various laboratory reports.

Let us consider the receiving station for a moment. The weight-dilution master card produced here contains all pertinent sample information. There is information that the laboratory needs, e.g., sample weight, dilution, and estimated potency. There is information that the accounting department needs, e.g., the number of the department submitting the sample and the unit charge for the assay. For some samples, such as retests and stabilities, there are special codes. In addition, there is a six digit code used to identify the sample as it passes through the laboratory from weighing bottle to volumetric to test tube. All of the data previously recorded on numerous laboratory data sheets is now entered on one card. Figure 2 shows

two keypunch operators punching the information that is recorded on the weight-dilution master card. As you can see, they operate right in the laboratory.

While the weight-dilution card is punched, the sample progresses through the laboratory in a group called a test. Each test consists of a standard, a possible 17 samples and media blanks arranged in two racks of test tubes. Each rack contains 10 rows of 4 tubes. The first row of four tubes represents the standard curve. The second row of four tubes represents the first sample and so on through 18 rows. In the nineteenth row, we repeat the standard curve and in the twentieth row we have our media blanks for adjusting the spectrophotometer. After a test is incubated, it goes to the reading station.

At the reading station (fig. 3) a punch card containing transmission readings is produced for every row, where a row is either a standard or a sample. This card must contain some means of sample identification so that it may be related to the weight and dilution necessary for the computation of sample potencies. This identification is the six digit code. The first two digits of the code identify the antibiotic or vitamin under test. The next two digits identify the test number and the last two digits locate the position within the test. The first four digits of this code are entered on a card through the IBM keyboards located directly above each spectrophotometer. For example, a 37-12 on the left keyboard indicates that the twelfth oxytetracycline test is being read while a 29-02 on the right keyboard indicates that the second vitamin B₁₂ test is being read. The position number, the last two digits of the code is entered automatically. The two keyboards enable a technician to read two tests concurrently, one on each spectrophotometer. Previously two technicians read and recorded the transmission readings of one test. This means that one technician now does the work of four.

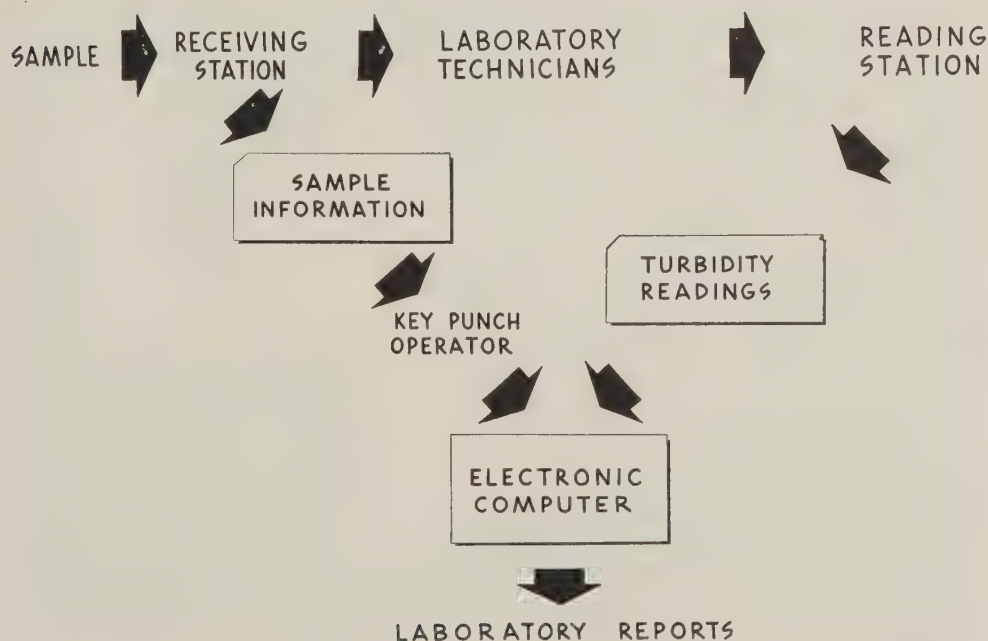


FIG. 1. Automatic production recording in our biological control laboratory.

After the code is entered on the keyboards, the instruments are set and a technician is ready to read. The mechanics of the reading operation are quite simple. A technician pours the contents of the test tube into the cuvette and presses the readout button on the keyboard. This is done first on the left and then on the right following the sequence of the position lights on the relay box. The relay box located between the spectrophotometers contains two sets of four white position lights and two red evacuation lights. These steady white position lights indicate whether the first, second, third, or fourth tube is to be read and the flashing of the red light indicates that the cuvette has been evacuated. If evacuation does not occur, the position light will not change signifying that something is amiss. Now let us consider how the equipment is functioning while the technician is emptying test tubes and depressing the two readout buttons.

Pressing the readout button activates the digital voltmeter, control console, and keypunches. The digital voltmeter is the unit used to convert the voltage output of the Spectronic 20 to a digital pulse acceptable for punching by the control console. The control console (fig. 4) is the intelligence of the system. It controls the punching of the date, keyboard information, position number and transmission readings on the IBM card. It also controls evacuation of the cuvette.

These instruments work together in the following way. After the readout button is pressed and the Spectronic 20 nulls, the digital voltmeter sends a pulse to the console to take a reading. The console sends a pulse to the keypunch and the light transmission reading is punched on a card. Then the console sends a pulse back to the relay box to evacuate the cuvette. One digital voltmeter serves both spectrophotometers, switching back and forth as the two readout buttons are depressed.



FIG. 2. Weight dilution master card keypunch operation.

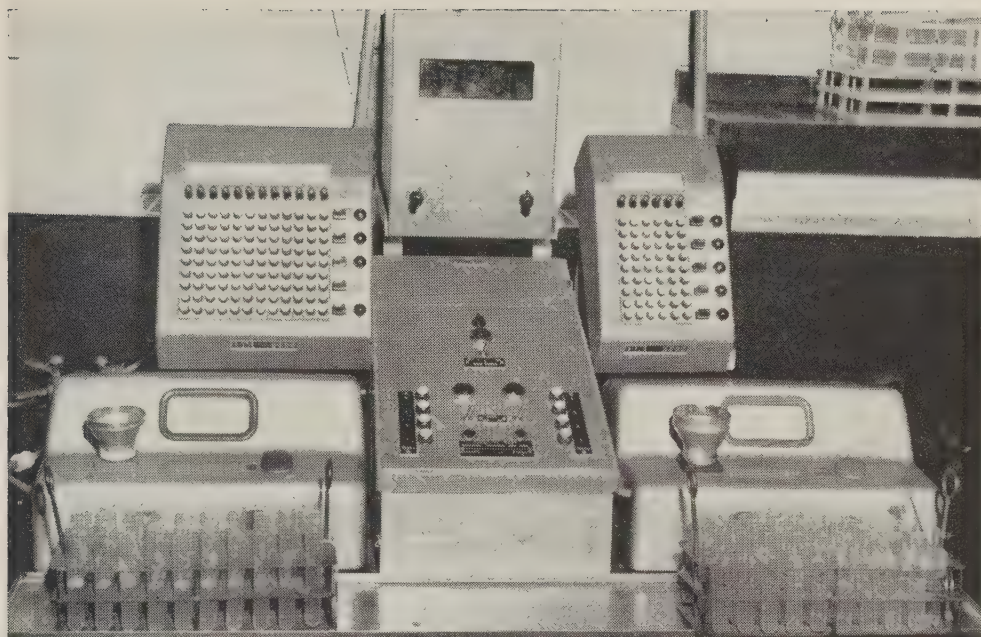


FIG. 3. Reading station: Two spectronic 20's, two IBM keyboards, a digital voltmeter, and a custom-built relay box.

If the digital voltmeter fails, transmission readings can still be entered on a card through the 12 column keyboard. Under this condition, a technician can only read one test at a time, thus we need only one 12 column keyboard.

The cards produced at the reading station and the weight-dilution master cards are fed to an IBM 650 electronic computer. It is not necessary to have the cards in any particular order because an IBM sorter, which operates at the rate of 1000 cards per minute, arranges all cards in order by antibiotic, by test, by position. The computer carries out 2000 program steps on each sample. Six hundred sample potencies are computed in just six minutes. It would take a laboratory technician 30 hours to accomplish this task with much less reliability.

The computational errors common to a manual operation no longer occur. Fitting a line to a set of points is somewhat arbitrary when the line is drawn by eye. Two technicians will seldom draw the same line through a given set of points. However, the computer selects that line which represents the best fit to the given set of points. The line is no longer arbitrary but precisely determined. Figures are no longer reversed, nor are they misread. There is no longer the "3" mistaken for an "8," the dilution 1000 computed as 100, or the case of the misplaced decimal. Besides the increase in precision and accuracy there are checks in the computational system that were not possible before. Extreme values are well defined and easily located. A point is not rejected by one technician because it looks too high and accepted by another who uses a different criterion for rejection.

After a thorough evaluation by the computer, sample potencies are reported back to the laboratory in the daily laboratory report (fig. 5). This report is produced from the computer output card which contains all of the information on the weight-dilution master card as well as the final potency.



FIG. 4. Reading station: IBM control console and two IBM remote punches.

The product code column contains the two digit product code preceded by a 1, which indicates that this is a turbidimetric test. For a plate test the product code will be preceded by a 2.

The department number, project number, and assay charge are used to prepare the monthly charge report for our Accounting Department. Previously each laboratory supervisor was forced to keep a daily running total of assay charges, since the accounting department required this report immediately at the close of an accounting period. Using IBM equipment to sort, accumulate, and print this report has saved our supervisors 40 hours per month.

The four columns headed by T, C, S, and R are special codes indicating the temperature of a stability sample, the number of capsules in a composite weight, the shipment number of a batch delivered in several parts, and a retest. The temperature code enables the IBM sorter to separate stability cards from all others after the daily laboratory report is completed. We then obtain a separate listing of stability results, which represents a considerable percentage of the daily samples. The laboratory supervisor no longer pages through a laboratory notebook recopying stability results for reporting. She merely sends out the listing prepared by IBM.

The column headed by P indicates the number of tubes on which the assay is based. In most instances, this is four.

The daily laboratory report which is filed as the permanent laboratory record actually takes about five minutes to list on the IBM tabulator. Other reports, such as the stability listing, are prepared in an even shorter period of time. These reports eliminate the handwritten laboratory note book, the necessity of sifting through pages for a particular class of results and recopying results for reporting.

DAILY LABORATORY REPORT													
DATE	PROD.	TEST	DEPT.	P*	CHG.	WT.	DIL.	SAMPLE IDENT.	T	C	S	R	EST. POT. P POTENCY AVERAGE
04/07/59	135	0102	7999	35	72	0.2501	2000	91211 / 42160					756 □/mg 4 736
04/07/59	135	0103	7999	35	72	0.2511	2000	91211 / 42160					756 □/mg 4 765
04/07/59	135	0104	7999	35	72	0.2522	2000	91211 / 42160					756 □/mg 4 753
04/07/59	135	0105	7999	35	72	0.2505	2000	91211 / 42160					756 □/mg 4 774 757

FIG. 5. A sample daily laboratory report is shown.

This completes a detailed description of the APR System. Let us briefly review the benefits of the system before we consider the new roads that it has opened to us. From an operational point of view, we have reduced by 20 per cent the personnel required to maintain the laboratory. We have replaced the 43 pieces of information previously recorded for every sample on 5 different data sheets by only 12 pieces of information entered on a punch card and automatically listed on one data sheet. Inaccurate transmission readings are now replaced by accurate readings, for the spectronic null point is now determined by the digital voltmeter and not by the

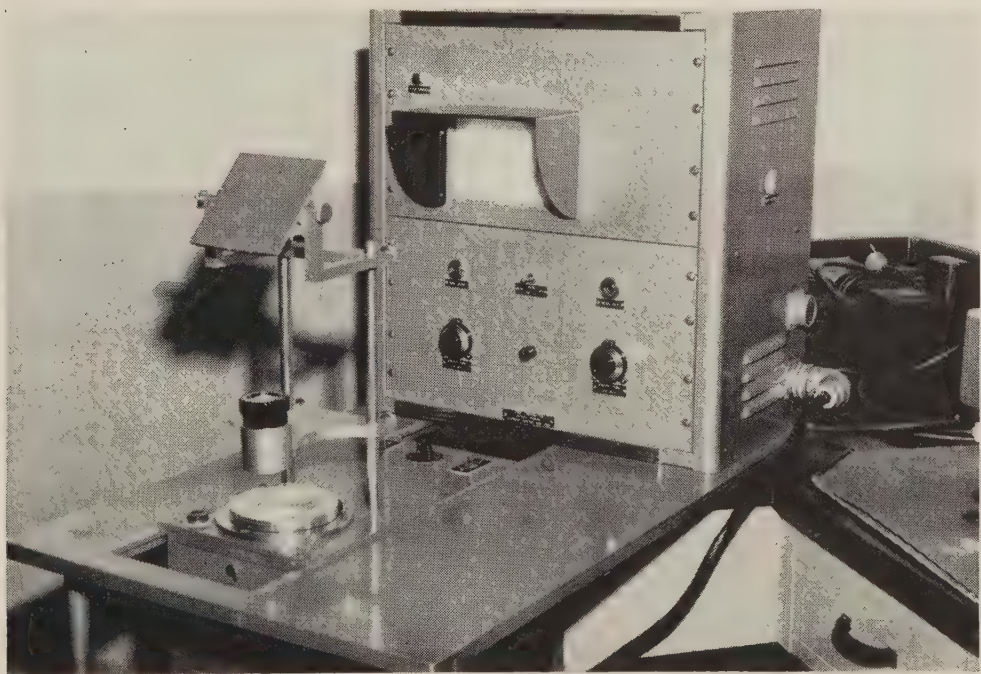


FIG. 6. An automatic zone comparator is illustrated.

decision of a technician. Common errors found in computing sample potencies are eliminated. The supervisors have been relieved of the burden of preparing the monthly charge report and recopying stability and production data for reporting.

From an assay point of view, we now have the option of using better designs, for we are no longer restricted by involved computations. The computer has solved this problem for us. We now obtain results based on precise computations while before many decisions were arbitrary. We have available information that was previously by-passed because the manpower for assembling the data was not available. It would not have been practical to do some jobs by hand that can now be done by machine in a matter of minutes. Perhaps we can sum up these benefits by saying that we get more reliable data, faster and for lower cost.

Not only have we mechanized our turbidimetric assay laboratory, but we have also developed a similar system for our plate diffusion assays. For the past two years we have been working on our automatic zone comparator (fig. 6) that will automatically measure the diameter of a zone of inhibition.

A push button controls the rotation of the plate holder while two photoelectric cells, one of which is stationary, locate the edges of the inhibition zones. The distance traveled by the moving cell behind the projection screen determines the diameter of the zone.

We are going to connect the zone comparator to another IBM console and card punch by a digital shaft converter. We shall produce a card containing zone diameters just as we produced a card containing transmission readings. This system will then parallel the automatic production recording unit in the turbidimetric assay.

But this is not all. At an ever-increasing rate, new equipment is being announced for recording, transmitting, and processing data. Only a few weeks ago, new equipment was introduced that will enable us to collate the results of pharmacological, sterility, and potency testing. All results will be stored on a magnetic tape from which the computer will instantaneously give us the status of in-process lots as well as preparing completed test notes. In addition, our data processing group has revealed that computation time next year will be one sixth of its present fantastic speed with the reports being prepared simultaneously with the computations.

Recent advances in the field of electronics present a great challenge. Where these new developments will lead is unknown, but from experience we know that monetary savings, increased efficiency, and a higher degree of accuracy are available to those who seek them.

Respirometric Assay of Nystatin in Animal Tissue

JOHN R. GERKE, JOSEPH GENTILE, HERBERT STANDER,* AND JOSEPH F. PAGANO

The Squibb Institute for Medical Research, New Brunswick, N. J.

The antifungal antibiotic nystatin^{1†} has been used effectively in the treatment of mycotic infections of man and in animal feeds for treating moniliasis and promoting growth.² These usages made imperative the development of sensitive methods for detecting nystatin in tissues.

The turbidity of tissue homogenates interfered with reading the end point of the tube-dilution body-fluid method of Pagano and Stander,³ even when methylene blue or tetrazolium dyes had been added as indicators. Nonturbid tissue extracts were prepared, but nystatin recovery from them was low and the method was tedious. A method that is not affected by turbidity was suggested by the studies of inhibition of respiration by nystatin.^{4,5} To handle large numbers of samples, we looked for a simple device to measure gas production. Fermentation tubes were tried but were not accurate because a variable amount of the gas escaped. Warburg respirometers were not suitable for use with a large number of samples. To meet our requirements, we developed a method using a hypodermic syringe both as a reaction vessel and as a manometer.

This paper describes the preparation of tissue standards and samples, the use of syringes as respirometers, and the results of experiments that demonstrated the utility of the method.

MATERIALS AND METHODS

Media. All media were sterilized for 15 minutes at 120 C. An inoculum broth was prepared consisting of: Penassay broth, dehydrated (Difco), 17.5 Gm./liter; glucose, 10 Gm./liter; yeast extract (Difco), 5 Gm./liter; tryptone, 10 Gm./liter; and distilled water. Inoculum agar for weekly transfers of the test organism consisted of inoculum broth with 1.5 per cent agar added. Assay broth was prepared with: Casitone (Difco), 9 Gm./liter; glucose, 70 Gm./liter; yeast extract (Difco), 5 Gm./liter; sodium citrate, 10 Gm./liter; potassium hydrophosphate, 1 Gm./liter; potassium dihydrophosphate, 1 Gm./liter; and distilled water. Just before use, to each liter of sterile assay broth was added 0.13 ml. of a dimethyl sulfoxide solution containing 4000 units of nystatin per ml. (final concentration, 0.5 unit/ml.‡) and to control bacterial contamination was added 1 Gm. each of penicillin and streptomycin.

Inoculum. The test organism was *Saccharomyces mellis* (Squibb 1647). A fresh

* Present address: Schering Corp., Bloomfield, New Jersey.

† The trade name of E. R. Squibb & Sons Division, Olin Mathieson Chemical Corp., for nystatin is Mycostatin.

‡ With some samples, levels of nystatin less than 0.5 unit/ml. enhanced gas production. The addition of 0.5 unit/ml. of nystatin causes partial inhibition of respiration; therefore, any further addition of nystatin cannot stimulate gas production, but results in increased inhibition.

TABLE I

Dosing of Tubes with 0.01 ml. of Nystatin Standard Solution: Nonfatty Tissue

Tube no.	Nystatin standard concentration, units/ml.	Final concentration, units/Gm. of tissue
1	16,000	160
2	4,000	40
3	1,000	10
4	250	2.5
5	(0.01 ml. of dimethyl sulfoxide)	0

stock slant was prepared each week on inoculum agar from the slant of the previous week and incubated for 24 hours at 37 C. From this slant, a 500 ml. Erlenmeyer flask containing 100 ml. of inoculum broth was inoculated and incubated for 17 hours at 37 C. on a reciprocal shaker (120 1½ inch strokes/minute). A 25 ml. aliquot of this inoculum was transferred to a second 500 ml. Erlenmeyer containing 100 ml. of inoculum broth and incubated for three hours at 37 C. on a reciprocal shaker. After three hours the flask was removed and stored at 5 C. until needed, but for not longer than one week.

Just prior to use, a 100 ml. aliquot of the inoculum was blended for 1½ minutes in a Waring blender to break up aggregates formed during incubation. The blend was centrifuged to concentrate the cells. The yield of cells was approximately 2 per cent (v/v). The supernatant was discarded and the cells resuspended in approximately 10 ml. of assay broth to produce a 20 per cent cell suspension.

Preparation of Tissues for Standard Curve. **NYSTATIN SOLUTIONS.** A 30,000 units/ml.* solution of nystatin in dimethyl sulfoxide was prepared. For use with nonfatty tissues this solution in dimethyl sulfoxide was diluted to each of the following concentrations: 16,000, 4000, 1000, and 250 units/ml. For use with fatty tissues the 30,000 units/ml. solution in methanol was diluted to the following concentrations: 3200, 800, 200, and 50 units/ml.

STANDARDS FOR NONFATTY TISSUE. The tissues were ground in a meat grinder before processing, the grinder being rinsed with methanol between samples.

A 10 Gm. portion of nystatin-free tissue was placed in a graduated cylinder and diluted to 95 ml. with assay broth containing 0.5 unit/ml. of nystatin. This was blended in a Waring blender for two minutes, and then poured into a flask. To dissipate the foam 2 drops of mineral oil were added and the flasks were placed at 5 C. for about one hour. Five ml. of the 20 per cent *S. mellis* suspension was added and mixed thoroughly. Ten ml. aliquots of the inoculated blend were pipetted into each of the five test tubes (1 inch × 4 inches) and the tubes numbered 1 through 5. The tubes were dosed with 0.01 ml. of nystatin standard solution as shown in table I.

STANDARDS FOR FATTY TISSUE. The tissues were ground in a meat grinder, the grinder being rinsed with methanol between samples. Five 2 Gm. portions of the ground, nystatin-free fatty tissue were placed in separate mortars and dosed with 0.1 ml. of nystatin solution as shown in table II.

* One mg. equals about 3000 units of pharmaceutical grade nystatin.

TABLE II

Dosing of Tubes with 0.1 ml. of Nystatin Solution: Fatty Tissue

Mortar no.	Methanolic nystatin standard solution, units/ml.	Final concentration, units/Gm.
1	3200	160
2	800	40
3	200	10
4	50	2.5
5	(0.1 ml. methanol)	0

Each portion was dried under vacuum (about 3 mm. of mercury) to remove the methanol (20 to 30 minutes). Each portion was washed with hexane three times, using 20 ml. for the first wash and 10 ml. each for the second and third washes. It was essential to mash the fatty tissue with a pestle during the first wash to effect intimate contact between the solvent and the fat. Each wash was centrifuged to precipitate suspended nystatin. The clear hexane supernatants were discarded. The contents of the centrifuge tubes and mortars were air dried to remove residual hexane. The tissue residue in each mortar was moistened with 2 drops of assay broth, 10 to 15 mg. of sand was added, and the mixture was ground to a paste with a pestle. The paste was suspended in 18 ml. of assay broth containing 0.5 unit nystatin per ml. This suspension was pooled with its respective three precipitates from the hexane washes. To this was added 2 ml. of 20 per cent cell suspension.

Preparation of Samples Containing an Unknown Quantity of Nystatin. Unknowns were treated the same as the zero concentration of nystatin (tube 5), as just described.

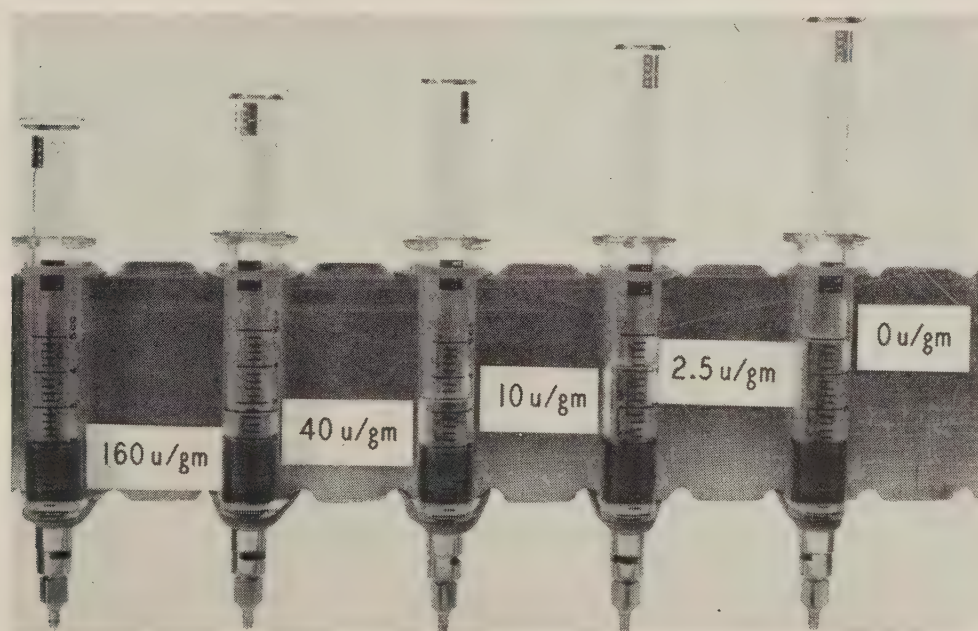


FIG. 1. Nystatin standard curve in syringe respirometers.

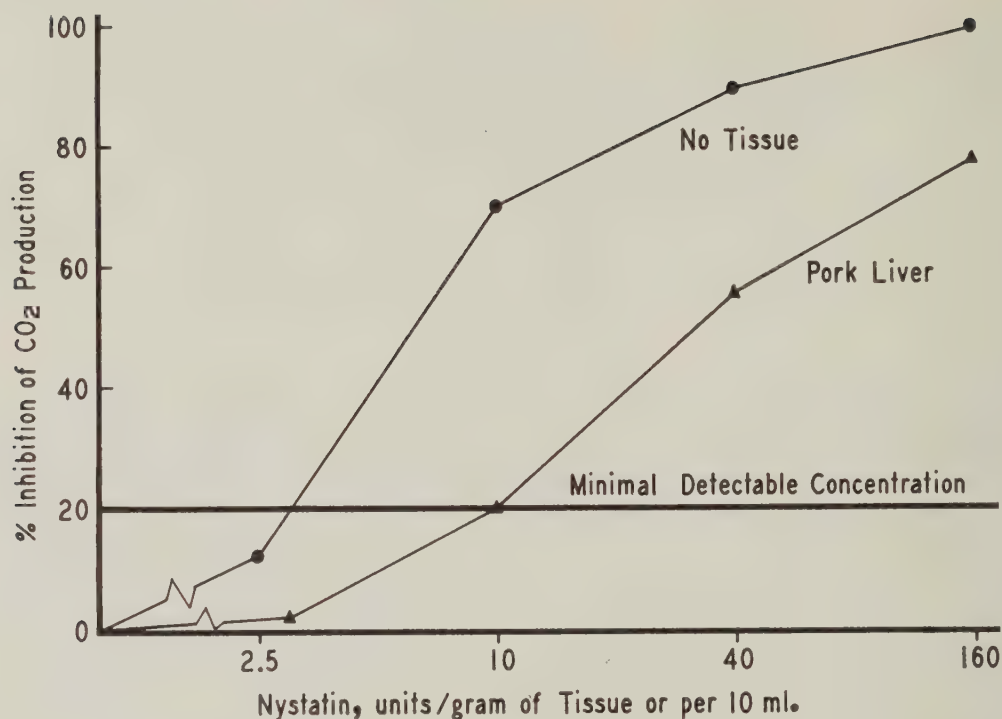


FIG. 2. Typical standard curves for nystatin are shown.

Syringe Assay. The plungers and barrels of 5 ml. Luer-Lok* syringes were lubricated with Blandol† or mineral oil of similar low viscosity to prevent gas leakage and to lessen friction. Two ml. of inoculated tissue suspension was drawn from each tube, as described previously, into a syringe (see figure 1) and capped with undrilled needle nub. The syringes were incubated in a 30 C. water bath. During incubation, the syringes were removed from the bath at 15 minute intervals and inverted several times to aid the release of dissolved carbon dioxide. The syringes were incubated until the syringe containing the 0 unit/ml. of standard produced approximately 3 ml. of gas. This required one to four hours, depending on the nature of the tissue. The volume of gas produced was read directly from the calibrations on the syringe barrel.

The standard curve was constructed by plotting carbon dioxide volumes versus logarithmic concentrations of nystatin, and from the curve, the concentration of nystatin in an unknown was read.

RESULTS AND DISCUSSION

Typical Curves, Reproducibility, and Sensitivity. As a practical test of this method, standard curves were run with swine and poultry tissues (see figure 2 for typical curves). Partial inhibition was obtained over a 50-fold range of nystatin

* Becton Dickinson & Co.

† L. Sonneborn Sons, Inc., New York City.

TABLE III

Minimal Detectable Concentration

Tissue	Number of determinations	Minimum detectable concentrations, units/Gm. of tissue		
		Average	Lowest	Highest
Chicken muscle	1	6.6	—	—
Chicken egg	4	4.5	1.7*	9
Pork liver	7	10	8	13
Pork kidney	3	8	5	10
Pork lean meat	1	8	—	—
Pork fat	2	12	10	13
No tissue†	6	3	1.5*	5

* Approximate values extrapolated from curve.

† Expressed in units/10 ml. to correspond to tissue concentrations of 1 Gm./10 ml.

concentrations. Since we expected to find little or no nystatin in actual tissue samples, we were concerned, primarily, with the end of the curve corresponding to the lower concentrations. The 95 per cent confidence limits of the volume of carbon dioxide produced in replicate determinations was ± 20 per cent. Therefore, a concentration of nystatin that caused a 20 per cent inhibition of carbon dioxide production differed significantly from zero concentration. This concentration was defined as the minimal detectable concentration under our experimental conditions.

Our results are summarized in table III. The minimal detectable concentrations varied from 3 to 12 units/Gm., depending on the tissue used. Due to the limited number of determinations, we were not able to ascertain if the variability was due to differences in the condition of the tissue or to minor differences in our experimental technique. In spite of this variability the study was useful because of the high sensitivity to nystatin in tissue. The variability shows the need for careful attention to technique and to the condition of the tissue used. Precision can be increased by replication.

Tissue Concentration. The minimal inhibiting concentration of nystatin in the body-fluid tube-dilution method of Pagano and Stander³ increased as the concentration of plasma increased. To assure conditions of maximum sensitivity for this respirometric method, the minimal detectable concentration of nystatin was determined for different concentrations of tissue. The results are shown in table IV.

TABLE IV

Minimal Detectable Concentrations in Different Concentrations of Tissue

Tissue	Concentration of tissue, Gm./10 ml. of assay broth	Sensitivity, minimum detectable concentration of nystatin	
		Units/ml. of assay mixture	Units/Gm. of tissue
Egg	2	1.24	6.2
	5	3.3	6.6
	10 (no assay broth)	6.2	6.2
Chicken Muscle	1	0.52	5.2
	2	1.04	5.2

As the concentration of tissue increased, the amount of nystatin required to inhibit respiration (minimal detectable concentration in units/ml. of assay mixture) increased. However, increased sample size did not affect the ability of the method to detect nystatin in tissue (minimal detectable concentration in units/Gm. of tissue). The lower tissue concentration, 1 Gm./10 ml., was preferred because it was generally easier to work with and consumed less tissue/determination.

Syringe Versus Warburg Respirometers. To assure that sensitivity was not limited by the use of syringes as respirometers, we compared results obtained with syringe respirometers and Warburg respirometers. The minimal detectable concentrations were equivalent, 4.5 and 4.0 units/Gm. of egg, respectively (average of four determinations each). Because of the simplicity of operation, we preferred the syringe respirometers.

SUMMARY

In this respirometric method for assaying nystatin in tissues, extraction of the antifungal agent was not necessary. A suspension of the test organism, *S. mellis*, was placed in direct contact with a homogenate of the tissue in a 5 ml. hypodermic syringe, which served as a reaction flask for the mixture and simultaneously as a manometer after its orifice had been sealed. The volume of carbon dioxide produced by the yeast in the syringe was inversely related to the nystatin concentration of the tissue. Equivalent results were obtained with a Warburg respirometer; however, syringes were simpler to operate.

A practical test of the method was made by adding nystatin to swine and poultry tissues. Depending on the tissue used, the minimal concentrations detected were 3 to 12 units/Gm. of tissue.

ACKNOWLEDGMENTS

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A Rapid Method for Detection of Combined Effects of Cysteine and Metal Ions on the Activity of Antimicrobial Compounds

EUGENE D. WEINBERG

Department of Bacteriology, Indiana University, Bloomington, Ind.

The activities of the majority of the presently known antimicrobial compounds are influenced by specific metallic ions and by metal-binding agents.¹ Of the metal-binding agents, cysteine has often been reported to suppress the action of antimicrobial substances; among the numerous compounds so affected by cysteine are captan,² citrinin,³ gliotoxin,³ hygromycin A,⁴ patulin,⁵ penicillin,³ pyocyanine,³ and streptomycin.⁶ Of the hypotheses proposed to explain the mechanism of action of cysteine on antimicrobial compounds, the most logical is that the amino acid affects the drugs indirectly by altering the kinds or amounts of free metallic ions in the extra- or intracellular environment. To test this hypothesis, the following study was undertaken.

MATERIALS AND METHODS

Bacterial Strains and Culture Medium. The test bacteria consisted of two stock culture strains of coagulase-positive and one strain of coagulase-negative *Staphylococcus aureus*. The culture medium employed in the study consisted of nutrient agar, which was prepared by dissolving in distilled water 0.5 per cent polypeptone (BBL), 0.3 per cent beef extract (Difco), and 2.0 per cent flake agar (BBL).

L-Cysteine, Metallic Ions, and Antimicrobial Compounds. Solutions of L-cysteine, of nitrates of each of eight metallic ions (Mg^{++} , Al^{+++} , Ca^{++} , Mn^{++} , Fe^{++} , Co^{++} , Cu^{++} , and Zn^{++}), and of each of five antimicrobial compounds (kojic acid, sodium dimethyl dithiocarbamate, 8-hydroxyquinoline, oxytetracycline, and penicillin) were prepared with demineralized water. The solutions of L-cysteine and of the metallic salts were neutralized, autoclaved, and stored at 4 C., when not in use. The solutions of antimicrobial compounds were either autoclaved or, in the case of heat-labile substances, filtered through sintered glass, and were stored at -10 C. when not in use.

Method of Testing the Combined Effect of L-Cysteine and Metal Ions on the Activity of Antimicrobial Compounds. To test the combined effect of L-cysteine and metal ions on the antimicrobial compounds, a combination of the double-gradient plate⁷ and of the strip-gradient plate⁸ methods was employed. The first or lower layer of each plate contained the metallic salt to be tested, the second or upper layer contained L-cysteine, and the filter paper strip placed on the inoculated agar surface in the direction of the double-gradient contained the antimicrobial compound. Single-gradient plates containing either the metallic salt or L-cysteine, nongradient plates containing the antimicrobial compounds on filter paper strips, and double-gradient plates with strips soaked in demineralized water were routinely included as controls.

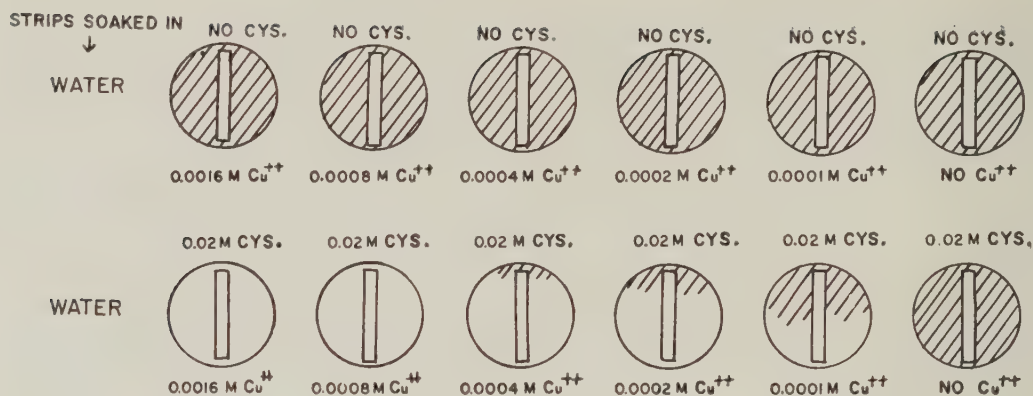


FIG. 1. Extent of bacterial growth on double-gradient plates containing varying quantities of cupric nitrate in the lower layer and of L-cysteine in the upper layer. The rectangles represent filter paper strips that had been soaked in water and placed on the agar surfaces at the beginning of the period of incubation. The shaded areas represent visible growth of *Staphylococcus aureus* after 24 hours at 37 C.

The concentrations of metallic salts and L-cysteine employed were usually the maximum quantities that were nontoxic to the test bacteria in single-gradient plates; however, in some of the tests to be described in the results, lower concentrations were employed. The concentration of each antimicrobial compound (except kojic acid) in which the filter paper strips were soaked was so adjusted that zones of inhibition between 35 and 50 mm. in width would be obtained on the nongradient control plates. With kojic acid the maximum soluble concentration was sub-bacteriostatic, so that no zones of inhibition developed adjacent to the strip that contained this compound. Sufficient viable cells to ensure an even carpet of growth were spread over the agar surface of each plate prior to the addition of the filter paper strips. Our best results were obtained with inocula of approximately 10^3 cells/plate.

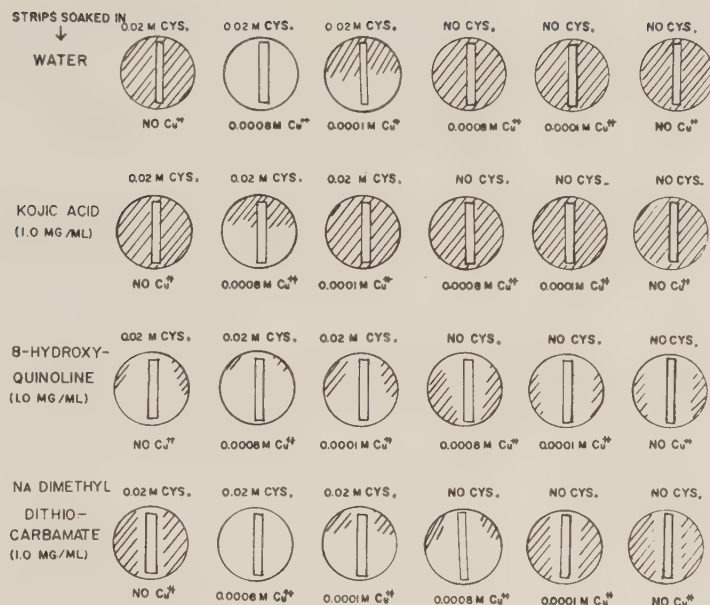
RESULTS

The results obtained with the double-gradient control plates containing strips impregnated with water indicated that the antibacterial action of Cu^{++} but not of any of the other seven metallic ions was enhanced by L-cysteine (fig. 1). In the absence of L-cysteine, a 25-fold concentration of Cu^{++} was required to obtain the same bacteriostatic effect that was achieved with the combination of amino acid and metallic ion. Molar ratios of L-cysteine to Cu^{++} of between 10 and 100 to 1 possessed maximum bacteriostatic activity.

It may be observed in figure 2 that sub-bacteriostatic concentrations of kojic acid were fairly effective in overcoming the bacteriostatic action of L-cysteine plus Cu^{++} . 8-Hydroxyquinoline was less active in this respect, and the carbamate was completely inactive in neutralizing cysteine- Cu^{++} toxicity. It may also be noted in figure 2 that the bacteriostatic action of 8-hydroxyquinoline was suppressed by high concentrations of Cu^{++} and enhanced by low concentrations of L-cysteine, whereas the carbamate was enhanced by Cu^{++} and not affected by L-cysteine.

In figure 3 it may be seen that L-cysteine strongly enhanced the ability of Fe^{++} to suppress the bacteriostatic action of oxytetracycline. In the absence of

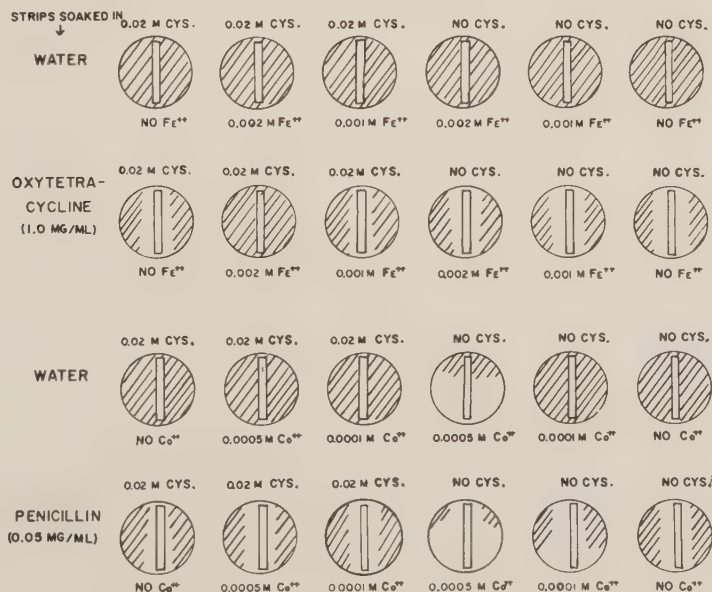
FIG. 2. The procedure is the same as in figure 1, except that strips contained antimicrobial compounds as indicated.



added Fe^{++} , L-cysteine had very little effect on the drug. Also in figure 3 it may be observed that the antibacterial action of penicillin was enhanced by Co^{++} (or was the antibacterial action of Co^{++} enhanced by penicillin?) and that L-cysteine suppressed rather than potentiated the effect of Co^{++} . In the absence of Co^{++} , the drug was slightly suppressed by L-cysteine.

The results presented in figures 1-3 were obtained with one of the test strains of coagulase-positive *Staph. aureus*. With the other coagulase-positive strain and with the coagulase-negative strain, comparable but not identical results were obtained.

FIG. 3. The procedure is the same as in figure 1, except that strips contained antimicrobial compounds as indicated and lower layers contained nitrates of iron or cobalt rather than copper.



Numerous examples of the intensification of the toxic action toward various types of biological systems of metallic ions by metal-binding agents have been reported. Some of these reports, as well as a theoretical discussion of the phenomenon, are contained in the excellent review by Albert.⁹ Additional examples of this phenomenon include the observations by Konowalchuk et al,¹⁰ who found that a reaction product of cysteine and Fe^{+++} possessed greater antitubercular activity than either the amino acid, the metal, or colloidal sulfur; by Woiwood,¹¹ who observed that an autoclaved product of cysteine and Cu^{+} (probably copper sulfide) was toxic toward gram-positive bacteria; and by Goodman,¹² who summarized reports of the enhanced antibacterial activity of combinations of streptomycin and various forms of copper. The enhancement of antibacterial action obtained by appropriate combinations of zinc and bacitracin was reported previously.¹³

In the present study, both potentiation and suppression by L-cysteine of bacteriostatic activity of metallic ions plus drugs were obtained. The most notable instance of suppression of bacteriostasis by L-cysteine occurred with ionic iron plus tetracycline, in which case the ability of ionic iron to protect the cells from the drug was considerably enhanced by the amino acid. It is suggested that whenever tests of metallic ions and the tetracyclines are performed, the possible influence of L-cysteine on the system be examined. Several additional examples of the favorable action of metal-binding agents and metallic ions toward biological systems have been cited by Albert,⁹ and it is also of interest in this connection that Cu^{++} has been found to enhance the ability of penicillin and chlortetracycline to stimulate growth of pigs and chicks.¹⁴

Although the method described in the present study for rapid detection of the influence of L-cysteine on various combinations of metallic ions and antimicrobial compounds was quite satisfactory, other methods might be equally efficient. For example, the method designed by Feeney et al¹⁵ in which, as in the present study, three substances are simultaneously tested (two metallic ions and a metal-binding agent) should be equally useful for tests with two metal-binding agents and one type of metallic ion.

SUMMARY

The ability of L-cysteine to influence the bacteriostatic activity of antimicrobial compounds in the presence of various metallic ions was tested by a combination of the double-gradient plate and the strip-gradient plate methods. The amino acid was found to enhance greatly the toxicity of copper, to enhance the ability of iron to neutralize oxytetracycline, and to depress the ability of cobalt to potentiate penicillin.

ACKNOWLEDGMENTS

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Single Disc Versus Multiple Disc and Plate Dilution Techniques for Antibiotic Sensitivity Testing

ALFRED W. BAUER, C. EVANS ROBERTS, JR.,* AND WILLIAM M. M. KIRBY

*Department of Medicine, University of Washington School of Medicine,
and King County Hospital, Seattle, Wash.*

Testing of bacterial susceptibility to antibiotics has been of increasing interest and importance because of the upsurge of infections due to antibiotic-resistant organisms. Methods employing drug-impregnated paper discs have been most widely used because of their simplicity. However, uniform standards are lacking and the results are considered by many to be unreliable because of a number of variables that are difficult to control.¹ One of these variables has probably been minimized by the recent establishment of control of disc potency by the federal Food and Drug Administration. Also contributing to uncertainty about the disc method is the fact that there are two procedures of testing in use, which frequently give conflicting results. These methods are the single and multiple disc techniques.

Historically speaking, disc diffusion was early used to measure penicillin concentrations, and it has remained a standard tool for antibiotic assays. It was later applied to sensitivity testing, and a number of workers have found a good correlation between zone sizes and bacterial susceptibility to a variety of antibiotics.²⁻⁵ The single disc technique is based on this principle.

The multiple disc technique was introduced as a simplification of the tube dilution test. A clear zone of inhibition around a given disc was assumed to represent an end point analogous to the minimal inhibitory concentration with the tube dilution test. Disc potencies comparable to antibiotic concentrations attainable in the blood stream of patients under therapy were selected, and the presence or absence of a zone of inhibition around two or three discs was used to indicate resistance or susceptibility. This point of view was adopted by manufacturers of dry commercial antibiotic discs, and the instructions available to most laboratories recommend the use of at least two discs. One manufacturer states, "presence or absence of a zone of inhibition surrounding a test disk and not the diameter or area of the zone should be considered to indicate susceptibility of a microorganism."⁶ The brochure of another manufacturer states that "a zone of inhibition of growth around sensitivity disks indicates that the organism is sensitive to the therapeutic agent."⁷ Measurement of zone sizes is discouraged because of variabilities in techniques from laboratory to laboratory.

Surprisingly, the literature contains no comparisons of the single and multiple disc techniques. The present study presents such a comparison and indicates that there is much better correlation between results obtained with the single disc and tube dilution methods than with the multiple disc and tube dilution techniques. A modified plate dilution technique recently described by Steers and associates⁸

Sensitivity discs for this study were generously supplied by the Difco Co.

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was also included in our study, both to assess its reliability and to compare its ease of performance with the single disc technique.

METHODS AND MATERIALS

Details of the single disc technique as performed in our laboratory have been presented in earlier publications.⁵ Discs of high antibiotic content (Difco) are used, and the tests are performed on Mueller-Hinton agar in 14 cm. Petri dishes, which makes it possible to test susceptibility of an organism to 10 or 12 antibiotics simultaneously. A technician can perform the test and read it the next day for a total time of about two or three minutes. Zone sizes of inhibition are measured with a mm. scale across their full diameter, and the results are interpreted according to standards published previously.⁵

For the multiple disc tests, discs of three potencies were used for each antibiotic, as follows: for tetracycline, 30, 10, and 5 $\mu\text{g.}$; erythromycin, 15, 5, and 2 $\mu\text{g.}$; chloramphenicol, 30, 10, and 5 $\mu\text{g.}$; streptomycin, 100, 10, and 2 $\mu\text{g.}$; and penicillin, 10, 5, and 2 $\mu\text{g.}$ The presence or absence of a zone of inhibition was recorded, and the results were interpreted as shown in table I. Although three discs were used, the results were the same whether the interpretation included the use of two or three discs. The remainder of the technique (such as inoculum size, incubation period, medium) was the same as for the single disc procedure.

The replicate plating method was carried out with a device described recently by Steers et al.⁸ This ingenious apparatus makes it possible to transfer separate organisms from 36 wells in a Teflon plate to a definite position on square antibiotic-impregnated agar plates. To prepare the plates, antibiotics were incorporated into tryptose blood agar in the following concentrations: tetracycline, 5 $\mu\text{g./ml.}$; erythromycin, 4 $\mu\text{g./ml.}$; chloramphenicol, 15 $\mu\text{g./ml.}$; streptomycin, 15 $\mu\text{g./ml.}$; and penicillin, 5 units/ml. A single concentration was used for each antibiotic. In performing the test, several colonies of each organism were transferred from an agar plate to a tube containing 3 ml. of tryptose phosphate broth and the tubes were incubated for 30 minutes, after which 0.1 ml. was transferred to one of the wells in the Teflon plate. A drop of this inoculum was then transferred to the antibiotic-containing agar plate with the replicating apparatus, and the presence or absence of growth was noted after overnight incubation.

TABLE I
Rules for Interpretation of Results of the Multiple Disc Test

High content disc	Medium content disc	Low content disc	Interpretation
No zone	No zone	No zone	Resistant
Zone	No zone	No zone	Slightly sensitive*
Zone	Zone	No zone	Moderately sensitive*
Zone	Zone	Zone	Sensitive

* For purposes of comparison with the other three methods, the two intermediate categories (slightly and moderately sensitive) are grouped together and referred to as "intermediate" in the subsequent tables.

TABLE II

Comparison of Results with 100 Strains of Staphylococci Tested by All 4 Methods

	Tube dilution method			Replicate plating method		Single disc method			Multiple disc method		
	S	I	R	S	R	S	I	R	S	I	R
Tetracycline	57	1	42	57	43	57	1	42	61	35	4
Erythromycin	73	0	27	73	27	73	1	26	89	5	6
Chloramphenicol	91	1	8	91	9	91	0	9	93	6	1
Streptomycin	50	0	50	50	50	45	6	49	59	32	9
Penicillin	22	7	71	23	77	22	5	73	89	7	4

S = Sensitive; I = intermediate; R = resistant.

The tube dilution tests were performed with an inoculum of 0.5 ml. of a 10^{-2} dilution of an overnight broth culture of staphylococci, added to 0.5 ml. of antibiotic in appropriate dilutions. Thus, an inoculum of 1 to 5 million organisms/ml. was used. Tryptose phosphate broth was the medium employed for the overnight culture and the remainder of the assay. The test tubes were incubated for 18 hours, and the lowest antibiotic concentration causing complete macroscopic inhibition of growth was recorded as the bacteriostatic end point, or minimal inhibitory concentration. Results were interpreted as follows: Minimal inhibitory concentrations of tetracycline or erythromycin that fell between 5.0 and 25 $\mu\text{g./ml.}$ were called "intermediate." Results above this range were resistant, and those below, sensitive. Similarly, the intermediate range for chloramphenicol and streptomycin was 25 to 50 $\mu\text{g./ml.}$, and for penicillin it was 2.0 to 50 units/ml.

The test organisms used in this study were 100 strains of coagulase-positive staphylococci recently isolated from patients at the King County Hospital.

RESULTS

In table II the over-all results of the four test methods with 100 strains of staphylococci are presented. Almost identical results were obtained with three of

TABLE III

Number of Instances Where the Results of the Single or the Multiple Disc Procedure Deviated from the Tube Dilution Test

	Disagreement, single disc test		Disagreement, multiple disc test	
	Complete	Partial	Complete	Partial
Tetracycline	0	0	3	36
Erythromycin	1	1	14	7
Chloramphenicol	0	1	3	6
Streptomycin	0	6	8	29
Penicillin	0	9	59	11
Bacitracin	0	0	0	0
Total	1	17	87	89

the four methods, namely, the tube dilution, replicate plating, and single disc techniques. With the multiple disc test, on the other hand, many more strains of "intermediate" susceptibility to tetracycline and to streptomycin were recorded. An even more striking discrepancy was the observation that many strains were interpreted as being "sensitive" to penicillin and erythromycin with the multiple disc method, whereas they were clearly resistant with the other three tests.

The results in table II do not indicate how frequently there was agreement or disagreement among the four methods, since any one discrepancy might have compensated for another. To clarify this point, the number of instances where the single disc or the multiple disc method yielded results deviating from the tube dilution test is shown in table III. For purposes of comparison, "resistant" with one test and "sensitive" with the other is recorded as "complete disagreement," while "intermediate" as opposed to "sensitive" or "resistant" is recorded as "partial disagreement." With the single disc method there was only one instance (with erythromycin) of complete disagreement among the 500 comparisons, and only 17 instances of partial disagreement (0.2 per cent complete, 3.4 per cent partial disagreement). In 96.4 per cent of the comparisons, the results were identical. With the multiple disc method, on the other hand, there was complete disagreement in 87 instances (17 per cent) and partial disagreement in 89 instances (18 per cent). The results were identical in only 65 per cent of the 500 comparisons of the multiple disc and tube dilution tests.

The nature of the errors encountered in using the multiple disc technique is illustrated in table IV. The test results and their interpretation are given for three groups of eight strains of staphylococci tested with tetracycline, streptomycin, and penicillin, respectively. For the first two strains with each antibiotic, the test results were identical by all three methods. With the next three there was "partial disagreement," and with the last three there was "complete disagreement." With tetracycline, it can be seen that strains with a minimal inhibitory concentration of 100 $\mu\text{g./ml.}$ with the tube dilution test showed zones of bacterial inhibition around all three discs of the multiple disc assay, around two, one, or none of them. However, only in the last instance is a strain correctly classified by the multiple disc method as resistant. With the single disc technique on the other hand, a small zone as well as no zone of bacterial inhibition indicates resistance, and much better results are obtained.

Discrepancies in test results are even more evident with streptomycin. For example, strains with zones of bacterial inhibition measuring 10 mm. about the single disc are classified as resistant, and grow in 100 $\mu\text{g./ml.}$ in tube dilutions, but exhibit clear-cut zones about two or all three discs of the multiple disc assay. With the latter method these strains would be called "sensitive" or "moderately sensitive," whereas with the other two methods they are resistant.

The most striking errors in classification were with penicillin, where despite the heavy confluent inoculum used throughout this study, resistant organisms (e.g., with minimal inhibitory concentration greater than 100 units/ml.) often produced zones of bacterial inhibition about all three discs with the multiple disc assay. Thus, they were resistant with the tube dilution test, resistant with the single disc test (zone sizes up to 23 mm. indicate resistance), but were "sensitive" with the multiple disc test. As shown in table III, this situation occurred in 59 of 100 instances and

TABLE IV

Comparison of Test Results and Interpretation for the

		Tetracycline, strain designation							
		1	24	91	96	20	71	73	74
Tube dilution test (minimal inhibitory concentration in $\mu\text{g.}/\text{ml.}^*$)		100	1	100	100	100	100	100	100
Interpretation		R	S	R	R	R	R	R	R
Single disc test (zone diameter, mm.)		6	30	8	8	8	9	8	8
Interpretation		R	S	R	R	R	R	R	R
Multiple disc test (disc content)									
	High	-†	+	+	+	+	+	+	+
	Medium	-	+	-	+	+	+	+	+
	Low	-	+	-	-	-	+	+	+
Interpretation		R	S	I	I	I	S	S	S

* The minimal inhibitory concentration with penicillin is indicated in units/ml.

† + means zone and - means no zone.

led to the erroneously high percentage (89 per cent) of penicillin-sensitive organisms with the multiple disc method (table II).

Except for a single instance, all strains called sensitive by the replicate plating method were also called sensitive by the tube dilution method, and vice versa. The lone exception was a strain with a minimal inhibitory concentration to penicillin of 5 units/ml., which failed to grow on the 5 units/ml. plate. This strain was therefore called "sensitive" with the replicate plating method, but "intermediate" by the tube dilution technique.

DISCUSSION

The present study indicates that, using currently recommended procedures and interpretations, the single disc technique with measurement of zone sizes is considerably more accurate as a means of determining bacterial susceptibility than is the multiple disc method. In addition, the single disc method is much simpler to perform, and is more economical, since it is possible to test 10 or 12 antibiotics on a single large culture plate.

The chief difficulty with the multiple disc procedure is that in some instances a clear zone of inhibition is present around a given disc even though the organism is resistant, but according to the recommended interpretations it is necessary to classify it as susceptible. This difficulty could be corrected by lowering disc antibiotic content to a point where it would just fail to inhibit the growth of resistant organisms. However, it is uncertain whether adequate standardization of the disc content could be achieved at such levels. In actual practice, experts familiar with the problem realize that a small zone of inhibition about a disc should often be regarded as no zone, and correctly designate an organism producing such a small zone as resistant.⁹ This practice is in effect a recognition of the importance of zone sizes. Unfortunately, laboratory workers not familiar with this refinement will read the results erroneously in a significant number of tests, as is clearly indicated in the present study.

TABLE IV

Two Disc Methods and the Tube Dilution Method

Streptomycin, strain designation								Penicillin, strain designation							
40	43	44	37	75	12	71	63	75	79	74	87	65	71	64	66
<100 R	5 S	<100 R	<100 R	<100 R	50 R	<100 R	<100 R	<100 R	0.1 S	<100 R	<100 R	<100 R	<100 R	<100 R	<100 R
6 R	19 S	10 R	10 R	9 R	17 I	13 I	10 R	6 R	38 S	8 R	8 R	13 R	16 R	21 R	21 R
-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+
-	+	-	-	-	+	+	+	-	+	-	-	-	+	+	+
R	S	I	I	I	S	S	S	R	S	I	I	I	S	S	S

Errors in interpreting results of the multiple disc test have their origin in a basic misconception, namely, that there is a useful correlation between disc content and blood levels. Such is not the case. An antibiotic diffusing from a disc into a semi-solid medium produces a gradient in concentrations from very high near the disc (much higher than the minimal inhibitory concentration as determined by a tube dilution test) to very low further out. It is for this reason that a definite zone of inhibition can occur with a resistant organism, and this is the explanation for the incorrectness of the widely disseminated notion that any zone of inhibition indicates susceptibility.⁷

Reliability of the single disc technique is enhanced by the fact that most pathogenic bacteria are either sensitive or resistant, with few falling into the intermediate range.⁵ To be worth the extra effort and expense, the multiple disc method would need to have clear-cut advantages in differentiating these groups, and it is evident from the present study that no such advantages exist. Indeed, after studying five or six different disc concentrations for several antibiotics, Greer et al concluded that "one disk is sufficient if concentrations and zone sizes are properly standardized."⁹

Results of the modified plate dilution technique correlated well with the tube dilution tests, and the replicating apparatus of Steers et al⁶ worked well in our hands. However, this procedure was more cumbersome to perform than the single disc technique and would appear to be practical only when large numbers of organisms need to be tested. A further limitation of this plate dilution technique is that the end point is determined with a single antibiotic concentration, whereas many measuring points are accessible with disc diffusion.

SUMMARY

Experience in our laboratory over a period of several years using a single high concentration disc for each antibiotic, with measurement of zone sizes of inhibition, has yielded excellent correlations with tube dilution tests and clinical results. This

is in contrast to the common recommendation that it is necessary to use two or three discs of varying concentrations and to ignore zone sizes. Surprisingly, studies comparing the two techniques are not available in the literature.

One hundred strains of staphylococci were tested for susceptibility to five antibiotics with serial dilution, single disc, multiple disc, and plate dilution procedures. The single disc test proved distinctly superior to the multiple disc method, from the standpoint of both accuracy and ease of performance. Reasons for errors with the multiple disc method were illustrated and elucidated. A modified plate dilution technique also gave results that correlated well with tube dilution tests, but was more cumbersome to perform than the single disc method.

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Standardization of Antibiotic Sensitivity Discs Inadequate Without Standardization of Methods and Interpretation

ARNOLD BRANCH, D. H. STARKEY, AND EDNA E. POWER

*Department of Veterans Affairs Hospital Laboratories, Lancaster,
New Brunswick, and Montreal, Quebec, Canada*

For a number of years we have been studying the subject of standardization of manufactured antibiotic sensitivity discs.¹⁻⁵ Such discs were found, on assay by the Biologics Control Laboratory in Ottawa, to be inconsistent in containing their labeled potency. Progress toward obtaining uniform test results from discs has been made in Canada and the United States by the application of Food and Drug Administration control regulations.^{6,7} Some other countries have attained similar control when the discs have been placed under the aegis of a national institute or authoritative specialist.

In the present paper we shall attempt to show that it is also necessary for us to use uniform methods and interpretations in order to obtain better correlation in reporting the results of antibiotic sensitivity tests.

METHODS AND MATERIALS

The agar well and tube dilution methods, standardized for hospital laboratories of the Canadian Department of Veterans Affairs as to media, inoculum, time of incubation, and so on,⁸⁻¹⁰ are used to compare disc results.

Organisms. Coagulase-positive staphylococci, isolated in the routine laboratory, were selected to represent the three categories of sensitivity referred to in previous communications (sensitive, moderately resistant, and resistant).

Discs. Penicillin discs were obtained from the manufacturers from countries listed in table I. The assays on all these discs have not been completed. Disc tests were performed using, in most cases, nine and, in some cases, five strains of staphylococci representing the three categories mentioned previously. In some cases single discs were used and in others two of low and high concentration, as recommended by the manufacturer.

In compiling all the tables with these discs of foreign manufacture, we have had to condense the results into the usual three categories employed by us, i.e., sensitive, moderately resistant, and resistant. This was necessary because in many instances the manufacturer's interpretation covered up to as many as six categories, based on small variations in the size of the zone of inhibition. However, in every instance the individual interpretation, as described by the particular manufacturer, was complied with. In certain cases, as mentioned previously, no zone was present with resistant organisms, while in others small zones were present with resistant organisms.

EXPERIMENT I

The results of the disc tests of the various manufacturers are given in table I. For

TABLE I

*Results of Testing Discs of 14 Manufacturers with Staphylococci**

Manufacturer	Sensitive, strain no.			Moderately resistant, strain no.			Resistant, strain no.		
	797	795	794	781	788	770	768	766	769
Group I									
Italian		41 S		19 MR	20 MR		12 R	13 R	
Finnish		24 MR		13 R	12 R		0 R	0 R	
French 1	*34 S	30 S	32 S	13 MR	15 MR	14 MR	0 R	0 R	0 R
Swiss	41 S	37 S	38 S	18 MR	18 MR	19 MR	0 R	0 R	0 R
Swedish	40 S	37 S	35 S	15 MR	15 MR	16 MR	11 R	11 R	12 R
Danish	40 S	40 S	35 S	21 MR	21 MR	22 MR	15 R	14 R	16 MR
French 2						19 MR	11 R	0 R	0 R
Hungarian		31 S		15 MR	15 MR		0 R	0 R	
Polish		39 S		21 MR	20 MR		0 R	0 R	
Group II									
German	33 S	30 S	26 S	10 S	8 S	11 S	0 R	0 R	0 R
English 1	22 S	23 S	27 S	0 R	12 S	12 S	0 R	0 R	0 R
Group III									
English 2	27, 32 S	23, 29 S	26, 31 S	10, 12 R	10, 13 R	10, 13 R	0 R	0 R	0 R
Group IV									
American 1	33, 38 S	21, 35 S	21, 30 S	9, 15 MR	12, 16 MR	10, 15 MR	0 R	0 R	0 R
American 2	22, 36 S	32, 34 S	30, 35 S	12, 16 S	14, 15 S	14, 15 S	0 R	0 R	0 R

* Figures indicate size of zone of inhibition in millimeters. Italics denotes discrepancy in category outlined. S = sensitive; MR = moderately resistant; R = resistant.

purposes of analysis they are divided into four groups: (1) single disc with size of zone of inhibition measured to indicate degree of sensitivity; (2) single disc and zone size not measured, i.e., any zone indicating sensitivity; (3) two discs of low and high concentrations with zone size measured to indicate degree of sensitivity; and (4) two discs and zone size not measured—any zone indicating sensitivity.

As seen in table I, the sensitive organisms show large zones of inhibition and the resistant very small or none. There is a third category defined by zones of inhibition distinctly smaller than those outlining the sensitive organisms.

In our tests the Finnish and English 2 discs failed to outline a third category. In the case of the former, there were no sensitive organisms and, in the latter, no moderately resistant organisms. The German and English 1 discs only indicated two categories, since organisms with small or large zones are both included as "sensitive."

The Danish, Swedish, Finnish, Polish, Hungarian, and Italian manufacturers allow small zones of a stated size as still indicating resistance.

Of the two American manufacturers, although the zone sizes are nearly comparable, manufacturer 1 interprets small zones (<12 mm.) as of no significance, so that organisms 781, 788, and 770, which produced such zones, are called moderately resistant. Manufacturer 2 interprets these same strains as "sensitive," considering the small zones as significant.

It is of importance to note that each of the three organisms in the moderately resistant category is classified as "sensitive," "resistant," or "moderately resistant," using the various manufacturers' interpretations. This discrepancy could be diminished by changing the interpretation in individual cases.

EXPERIMENT II

This experiment was similar to experiment I, but 25 staphylococcal strains were

used and the results given as interpretations and numbers in each category by each type of disc (table II).

Group I. SINGLE DISC AND SIZE OF ZONE OF INHIBITION USED TO INDICATE DEGREE OF SENSITIVITY. Comparing our agar well results and those obtained using discs of four European distributors the results were good in indicating sensitivity. With penicillin, the most difficult thing is to differentiate between moderately resistant and resistant organisms. This was accomplished with fair success, except with the Danish discs. The concentration in this disc, 25 units, may be too high. It is only fair to add that the results of testing two moderately resistant strains by our method differed slightly from those obtained by the manufacturer in Switzerland using his method. This is probably due to a different inoculum size and the type of medium used, which are important factors.

Group II. SINGLE DISC WITH ANY ZONE INDICATING SENSITIVITY. This method did not prove satisfactory, since it is important to differentiate an intermediate category between sensitivity and resistance. Some organisms in this category have been shown to be treatable by large dosage of antibiotic.

Group III. TWO TABLETS AND ZONE SIZE MEASURED. The minimum size given by the manufacturer for a zone to be significant is 16 mm. It will be noted that our tests failed to differentiate between resistant and moderately resistant organisms. One reason for this may be that the high concentration tablet would give more satisfactory results if it contained a higher concentration of antibiotic. We sent the manufacturer two strains of penicillinase-producing staphylococci, and the results of tests are given in table III.

Group IV. TWO DISCS OF LOW AND HIGH CONCENTRATION AND ZONE SIZE NOT MEASURED. As pointed out previously, the results of American manufacturer 2 may be due to incorrect interpretation, since small zones of 12 mm. are not discounted. The results would be improved if an interpretation similar to that of the

TABLE II

*Results of Testing 25 Strains of Staphylococci with Penicillin
Discs of Various Manufacturers Using Given Technique*

	Sensitive	Moderately resistant	Resistant
Agar Well	10	8	7
Group I			
French	9	8	8
Swiss	11	7	7
Swedish	10	9	6
Danish	11	12	2
Group II			
German	19		6
English 1	16		9
Group III			
English 2	9	1	15
Group IV			
American 1	8	9	8
American 2	18	2	5
	(11)*	(7)	(7)

* Interpretation discounting very small zones as indicating sensitivity.

TABLE III

*Manufacturers' Results Compared with Our Results Using Same Penicillin Discs
but Our Techniques and Interpretations*

Manufacturer	<i>Staphylococcus</i> 781*				<i>Staphylococcus</i> 788*			
	Manufacturer's results		Our results		Manufacturer's results		Our results	
	Reading	Interpretation†	Reading	Interpretation‡	Reading	Interpretation†	Reading	Interpretation‡
Group I Swiss	(1) 20-22	Moderately sensitive			(1) 15-16	Weakly sensitive		
	(2) 17	Weakly sensitive (treatable)	18	MR	(2) 14	Very weakly sensitive (untreatable)	18	MR
Swedish	25	Moderately sensitive (treatable large doses)	15	MR	15	Slightly sensitive (treatable certain conditions)	15	MR
Danish	11	Relatively resistant (treatable)	21	MR	14	Relatively resistant (treatable)	21	MR
Group II German	Zone	Sensitive (treatable)	10	S	Zone	Sensitive (treatable)	8	S
Group III English 2	12, 16	Partially resistant (untreatable)	10, 12	R	11, 15	Partially resistant (untreatable)	10, 13	R
Group IV American 1	0, 10	Resistant (untreatable)	9, 15	MR	0, 10	Resistant (untreatable)	12, 16	MR
American 2	14, 16	Sensitive (treatable)	12, 16	MR	14, 17	Sensitive (treatable)	14, 15	MR

* Both staphylococci moderately resistant by agar well method.

† As personally reported by manufacturer.

‡ Based on interpretations from manufacturer's brochure adapted to three categories. Readings given in millimeters. S = sensitive; MR = moderately resistant; R = resistant.

first manufacturer were used, as shown in the figures appended in parentheses in table II.

EXPERIMENT III

In order to compare our results using the discs of manufacturers listed in table III with those of the manufacturers themselves using their own methods and discs, we circulated two strains of moderately resistant staphylococci to them all. In this way, we expected to be able to assess the influence of certain variables, such as type of medium and size of inoculum.

Table III summarizes the findings of the manufacturers using their own disc and methods as against our findings using their discs and our methods. The interpretation given by the manufacturers is their own, while ours has been modified to fit into one of the three categories we employ, as outlined previously. Several points are obvious.

With group I, the variation in zone size between our tests and the manufacturer's shows wide variation. However, the interpretations agree, since there is a wide range in zone size to delineate the various categories.

The single disc in group II gave zones of 8 to 10 mm., with our test. The manufacturers did not report their zone size, merely reporting the organisms sensitive. Should not the interpretation be changed so that zones of inhibition of this magnitude be regarded as moderately resistant? (See table I.)

The English tablets in group III produced smaller zones with our tests than with the manufacturer's. Their interpretation is partially resistant and ours is resistant.

The American results in group IV are particularly interesting. With American manufacturer 2, the zone sizes practically agreed with ours, but the interpretation of the organisms is sensitive, while we considered them moderately resistant. With American manufacturer 1, our zone sizes were larger and caused a difference in interpretation.

As requested, the manufacturers, when forwarding the results of the tests, stated whether they considered these organisms treatable with penicillin. Five replied that they considered infections with strain 781 treatable, and there were a few similar replies with strain 788.

DISCUSSION

In analyzing the reasons for the afore-mentioned discrepancies, we should like to emphasize again the importance of the size of the inoculum in changing the size of the zone of inhibition.¹¹

The chance of the medium having some effect has latterly been minimized, but we would draw attention to two observations that show that it is not entirely without effect: (1) one American manufacturer reported results obtained using two different agars, and this caused a variation in zone size of 4 to 6 mm.; and (2) Dr. Chabbert, of the Pasteur Institute, has sent us detailed experiments to show that occasional batches of media prepared in the Institute may show much smaller zone sizes than other batches of the same media, particularly when testing tetracyclines. He also found that with polymyxin there is a significant variation in zone size with different batches of manufactured media. The other factors involved in producing discrepancies are faulty methods of interpretation and the lack of adequate concentrations of antibiotic in the discs to give the desired result.

The results obtained with discs in group I, i.e., a single disc and size of the zone measured to indicate degree of sensitivity, proved satisfactory. Experienced workers who use one disc with uniform concentration and standard methods can attain a high degree of correlation with other manufacturers of single discs.

SUMMARY

Control of the antibiotic content of manufactured discs is essential for reproducible results in sensitivity tests. Also, variations in the interpretation of results may give a false impression in terms of whether an infection with such an organism is really treatable. Differences in methods for reading results, as well as differences in media and size of inoculum affect the results. The size of the inoculum is of particular importance with penicillin when testing staphylococci.

RECOMMENDATION

In addition to the standardization of disc contents in terms of labeled potency, we recommend that further steps be taken to promote agreement in respect to details of technique.

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Antibiotic Susceptibility Testing by the Disc Method

THEODORE G. ANDERSON AND ANNA TROYANOSKY

*Associate Professor of Microbiology, and Temple University School of Medicine,
Philadelphia, Pa.*

The present status of disc susceptibility testing indicates that there is a definite need to standardize the in vitro method for the determination of the susceptibility of microorganisms when dehydrated antimicrobial impregnated paper discs are used. Other in vitro procedures¹⁻⁷ may be quantitatively more accurate than the disc method; however, because of simplicity, increased number of antimicrobials, economy, demand by physicians, and recognition^{8,9} that the disc test properly done and interpreted has value as a therapeutic guide, this practical method is used in the majority of routine diagnostic laboratories.

If the result of the quantitative disc test is accepted as a useful guide⁸ to therapy, then this test plays an important part in the direction of specific therapy today. Therefore, a method or a composite method should be derived from published procedures,¹⁰⁻¹⁵ analyzed, approved, sponsored, and accepted as the standard disc test method.

The results of a disc test are expected and accepted to be only an index of in vitro susceptibility without positive in vivo correlation. As an index, however, the result from a test properly done may be helpful; if improperly done, harmful. Present day antibiotic therapy did stem from an in vitro observation.¹⁶

The lack of confidence in disc testing has arisen from an attempt to interpret the test on an absolute quantitative basis, from a variety of methods of application and interpretation that have appeared in the literature, from attempts to use the results of the disc test on a comparative basis, and because there is no accepted recognized standard procedure.

An example of the chaotic state of disc testing may be found in the study reported by Hoffman et al.¹⁷ In their study one among nine teaching hospitals in the Chicago area, to whom cultures were distributed, was apparently interpreting the results of the disc test in the labelled micrograms of potency on the commercial discs. One can only feel with the authors that with such a bizarre event happening there could be little confidence in the disc results or hoped for correlation in their study.

In the first half of this year a survey¹⁸ was made of disc testing methods being used in hospital laboratories in Washington, Philadelphia, New York, Boston, Chicago, St. Louis, and Pittsburgh. Data were collected from 50 hospitals. All were using commercially prepared discs. A study of the data collected revealed that, when one incorporated methods of interpretation as a difference, 20 variations of disc testing were being used.

In the majority of instances among the 50 hospitals a single test disc was being used. However, one group was using only low concentration discs; another group, only the high concentrations. Some laboratories were using low potency discs of one antibiotic and an intermediate potency of another. It was also found that a low potency disc of one antibiotic would be used and the high potency disc of another.

TABLE I
Filter Paper Disc Standardization of Oxytetracycline

Organism	Tube* susc.	Disc zones (mm.) and concentrations (μg./ml.)									
		1000	900	800	700	600	500	400	300	200	100
<i>Staphylococcus</i> H	0.6	24	24	24	25	24	24	22	22	21	21
<i>E. coli</i>	0.5	—	—	—	—	—	25	—	—	—	—
<i>E. coli</i>	2.0	—	—	—	—	—	16	—	—	—	—
<i>E. coli</i>	5.0	—	—	—	—	—	14	—	—	—	—
<i>E. coli</i>	9.5	—	—	—	—	—	10	—	—	—	—
<i>Proteus</i> sp.	10.0	—	—	—	—	—	0	—	—	—	—

* Minimal inhibitory concentration in μg./ml.

The two disc method as described in some manufacturers' brochures was being used in six hospital laboratories. One laboratory was using a low, intermediate, and high potency disc of each antibiotic.

Interpretation was as varied as methodology and ranged from any zone at all being called susceptible to measuring zone sizes with a set of values for susceptible, moderately susceptible, and resistant.

These findings further stress the need, as had been expressed,¹⁹ for an acceptable standard disc method if reproducible, reliable, and informative disc testing is going to be done.

In our laboratory for the past 10 years we have been using a single low potency disc method. This procedure is an adaptation of the "wet" disc method of Bondi et al.¹⁰ In this method Whatman No. 2 filter paper discs 6.5 mm. in diameter are saturated in a standardized antibiotic solution. The disc saturated with the standardized solution will produce a maximum large zone with an organism inhibited by a low concentration of antibiotic in a test tube titration with an inoculum of 0.5 ml. of a 1 to 1000 dilution of a 24 hour culture in 0.5 ml. of broth containing exactly measured quantities of the antibiotic. Organisms of lesser test tube susceptibility will present smaller zones and resistant organisms no zones with the disc saturated in the standardized solution (table I).

Table I shows the standardization of oxytetracycline for use in this method.

The saturated 6.5 mm. discs take up approximately 0.005 ml. of solution. The disc therefore contains approximately 2.5 μg. in this example for oxytetracycline.

Discs from the tubes containing greater concentrations of antibiotic than the 500 μg./ml. tube though they contained greater concentrations of antibiotic showed no marked increase in zone size with the most susceptible organism. At the 2.5 μg.

TABLE II
Interpretation of Disc Test Results with Low Potency Antibiotic Discs

Diameter of zone sizes	Interpretation
Greater than 15 mm.	Susceptible
10 to 15 mm.	Moderately resistant
Less than 10 mm.	Resistant

disc level (500 µg./ml. solution) the organisms that were more resistant by the test tube method showed decreasingly smaller zones. With antibiotic solutions of 2000, 6000, and 12,000 µg./ml. and discs correspondingly of approximately 10, 30, and 60 µg. potencies, zones with organisms susceptible to 0.5 µg. in the test tube, were of the order of 28 to 35 mm. in diameter. The organisms susceptible to 5, 10, and 15 µg. in the test tube zones were of the order of 20 to 25 mm. in diameter.

The concentrations of the standardized solutions for other antibiotics will be found elsewhere.²⁰

In interpreting the results with the standardized low potency discs, unfortunately some measuring of the zone size has to be done. The amount of measuring, however, is minimal (table II).

The interpretation as indicated in table II will apply to all commonly used antibiotics except polymyxin, bacitracin, ristocetin, and kanamycin and the drugs nitrofurazone and methenamine mandelate. With these six antimicrobial agents, any zone of inhibition around the disc identifies an organism as susceptible. The interpretation with nitrofurazone and methenamine mandelate applies only with respect to the use of these drugs therapeutically in urinary tract infections. The sulfonamide drugs are not included in our scheme of disc testing because the results of in vitro tests with these drugs are questionable when done on the ordinary media used in most diagnostic laboratories. With special media free of inhibitors, disc tests with sulfonamides apparently have value. However, if the physician is inclined to use a sulfonamide, clinical trial is of greater value than a disc test or test tube result. A negative in vitro test with a sulfonamide can be misleading. It should not be forgotten that if Domagk²¹ had not originally used in vivo methods with the sulfonamides their potential as a therapeutic agent possibly never would have been discovered. For those who wish to do in vitro sulfonamide susceptibility testing, inhibition of the test organism is an index of susceptibility when the low potency discs are used.

The susceptibility of *Mycobacterium tuberculosis* to isoniazid and *p*-aminosalicylic acid is preferably done by incorporating the drugs in a solid medium.²²

In any in vitro susceptibility test the most significant variable is the inoculum size. We have found a 4 mm. film loop inoculum of an 18 to 24 hour broth culture spread evenly over the surface of the plate to be most satisfactory. To assure an even distribution of organisms, the inoculum is applied by first streaking four to five times across the diameter of the plate, then completing the inoculation with overlapping streaks horizontally across the primary inoculation to cover the entire plate surface. A satisfactory plate inoculation may also be made using a standard swab. Dip the swab into the broth culture, express the excess out on the inside of the tube and streak the plate as previously described. An inoculum directly from colonial growth on the primary plate from the pathologic specimen may also be used. Select not less than three isolated colonies and using a loop incorporate growth from each colony in the inoculum. If there is a pure culture growth on the initial plate the inoculum may be taken from the mass growth on the plate. Streak the inoculum evenly over the plate surface as previously described. If a single colony is picked from a plate for either direct inoculation or for the preparation of a broth culture there is always the chance of selecting a resistant mutant from an otherwise susceptible strain and vice versa.

Referable to the inoculum, discs may be also applied to the initial area of inoculation on the plate^{9,10} streaked with the pathological specimen. In our method of inoculating plates with the primary specimen only six discs are used: three on the aerobic plate and three on the anaerobic plate. Streptomycin or dihydrostreptomycin are less active under strict anaerobic conditions and therefore should not be used on the anaerobic plate if exacting anaerobic culture methods are used. If partial oxygen reduction constitutes the anaerobic method, streptomycin or dihydrostreptomycin discs may be used. In no instance should one use discs on a plate inoculated with the pathological specimens in an area in which the inoculum is diluted by the streaking procedure. The use of this method of putting discs on the initial plate is debatable. However, in our hands we have found it time saving and effective. Care must be taken in interpreting the results. If the initial growth is very heavy or mucoid or if the growth is sparse, the test must be done from the pure subculture.

In any disc interpretation hazy, fuzzy, or spiculate zones may be seen. Zones of this type are common with the more bacteriostatic antibiotics. These zones are difficult to interpret even when interpretation is directed as susceptible or by any zone at all. It is preferable and suggested to submit a tentative report of doubtful results, as resistant or moderately resistant, pending a subsequent recheck of the equivocal test. With hemolytic organisms, an unhemolysed area may be seen around the disc. This area should not be interpreted as the zone of inhibition without careful examination, colonies may be found growing up to the disc in the zone of no hemolysis. This observation is not referable to the rapid hemoglobin reduction method,⁴ with which the authors have not had any experience.

Providing the precautions mentioned are taken into consideration, *in vitro* disc testing, especially to determine the susceptibility of microorganisms to antibiotics, can be done in the routine diagnostic laboratory with the low concentration antibiotic discs and afford the physician information that may be helpful as a guide to therapy. The value of the *in vitro* disc test as a positive guide to successful therapy is debatable. There is, however, a good degree of correlation as many studies have shown, especially when the test is properly applied and interpreted.

The standardization of low potency discs for *in vitro* testing as described was done with so-called "wet" discs. We have run comparative tests with commercial low potency dehydrated discs and have found that in our hands and interpreting our results as already described there is excellent correlation between the "wet" and low potency dry disc results. Therefore, we now routinely use low potency dehydrated commercial discs. Because of our volume of testing, discs are applied with the dispenser, which we have found to be practical and to expedite the work in our laboratory where we annually process between 25,000 and 30,000 pathological specimens per year, on the majority of which susceptibility tests are done. In using the dispenser the discs must be touched down to the surface of the plate with a forceps after application to be sure the discs rest flat and firm on the agar surface. If this simple step is not done, uneven zones may result and discs will fall off the agar surface when the plates are inverted for incubation.

The antibiotics and drugs and the disc concentrations we use are shown in table III.

We use only the dihydrostreptomycin disc because there is no indicated difference in susceptibility between streptomycin and dihydrostreptomycin. We also use

only the tetracycline disc routinely because there is no significant difference in susceptibility among the tetracyclines. In certain problem cases we find it necessary to resort to test tube susceptibility tests. We feel that for infections, such as in the urinary tract, where there is a concentration of antibiotic, or drug, an organism may be considered to be susceptible that would be called resistant if the same organism were the etiologic agent in a blood stream or pulmonary infection. Therefore, organisms isolated from urine, for example, may be tested with the higher concentration discs. This is only an hypothesis and it would appear always advisable for the physician to use that antibiotic or drug to which the organism is most susceptible in vitro if toxicity and patient idiosyncrasy does not further limit its use. The laboratory in final analysis is only a source of information: the choice is the physician's prerogative. Results, however, should be as accurate and informative as possible.

TEST RESULTS

The literature is continually referring to the many variables that cloud disc testing results. We have investigated the following: type of basic medium for preparing blood agar plates, inoculum size, pH, depth of medium in the standard Petri plate, concentration of added blood, interval between plate streaking and disc application, and moisture.

Media. None of the generally used blood agar base media will significantly alter the zone size to give a misleading interpretation. We believe that disc tests should not be done on selective or differential media, such as E.M.B., SS, PEA, or tellurite because factors in the medium may be inhibitory to the organism, the antibiotic, or both. If Mueller-Hinton or any one medium is used for testing one organism or any one antibiotic, it should be used for testing all antibiotics and organisms. Media that permit greater antibiotic or drug diffusion are not necessarily more applicable to disc testing unless other media completely inhibit diffusion. The basic blood agar medium we use is Trypticase soy agar: we have not observed antibiotic inhibition in this medium as previously reported.²⁴

Inoculum Size. Light inocula are more misleading than heavy inocula. Large zones may be seen with penicillin and coagulase positive, penicillinase positive staphylococci with a light inoculum. Test tube titrations with strains of staphylococci as described may show they require 100 to 1000 units/ml. of penicillin to

TABLE III

Dehydrated Discs and Potency Used in Low Potency Disc Susceptibility Testing

Drug	Potency	Drug	Potency
Bacitracin	2 units	Dihydrostreptomycin	2 µg.
Chloramphenicol	5 µg.	Tetracycline	5 µg.
Erythromycin	2 µg.	Ristocetin	10 µg.
Neomycin	5 µg.	Kanamycin	5 µg.
Novobiocin	5 µg.	Nitrofurazone	100 µg.
Penicillin	2 units	Methenamine mandelate	1 mg.
Polymyxin	50 units	Sulfonamide*	1 mg.

* When tests are done on this drug.

inhibit them when an inoculum of 0.5 ml. of a 1 to 1000 dilution of the strain is used in the test tube titration.

pH. The pH range of media commonly used in the routine diagnostic laboratory is 6.8 to 7.4. Within this range we have not seen differences in zone sizes that would be significant relative to interpretation.

Amount of Medium per Plate. Zone sizes will not be significantly different in plates containing 10, 15, or 20 ml. of medium. Generally 12 to 15 ml. of medium is used per plate.

Concentration of Blood. Blood agar plates generally contain 3.0 to 5.0 per cent added whole defibrinated or citrated blood. We have found no significant alteration of zone size to give misleading results with blood concentrations up to 10 per cent with either defibrinated or citrated human or horse blood. We do not routinely use human blood because of the potential of inhibition for organisms in such blood.

Time of Disc Application. In the routine laboratory discs may be applied to the plates immediately after streaking or where the volume of work is large there may be a time lapse between streaking and disc application. We have found no significant differences in zone size between discs applied immediately after streaking and at 15 minute periods up to three hours.

Moisture. The amount of moisture on or in the medium has been considered a variable in disc testing. We have used freshly prepared and 24 hour old plates without significantly altering the zone size. We have also applied saline and distilled water to dehydrated discs placed on blood agar plates without inducing a misleading interpretation.

Comment. In these tests differences were not considered significant unless they would materially alter the zone size and place an interpretation in an entirely different and misleading category. In other words, "susceptible" organisms did not become "moderately resistant" or vice versa, and "resistant" organisms did not become "moderately resistant" or "susceptible." Differences in zone size in disc testing of 1 to 2 mm. are within the range of possible experimental error.

It is certain that when interpretation involves measuring zone sizes in establishing categories of susceptibility there is a twilight zone between categories where equivocation enters in. For example, is an organism presenting a zone of 10 mm. according to our method of interpretation "resistant" or "moderately susceptible," or on the other hand is an organism presenting a 15 mm. zone "moderately resistant" or "susceptible"? In our scheme borderline results are interpreted in the next lower category. Thus the borderline "susceptible" becomes "moderately resistant" and the "moderately resistant" borderline species becomes "resistant." This type of interpretation in which the physician has latitude in his therapeutic choice has intent toward the best in vitro suggested selection. When the results of an in vitro test are equivocal or if there are other reasons to doubt the results, the test should be repeated, possibly by test tube titration. In this area of equivocation the two tube test of Schneierson⁶ could be helpful in arriving, with less work, at a relatively more exact interpretation.

DISCUSSION

Disc testing is routinely being done with little apparent organized methodology

and in some areas with little thought. A method using low potency discs that we have found applicable for in vitro testing through the past 10 years has been described and a workable method of interpretation pointed out. It is not hoped that this procedure could remove all the stigma against disc testing nor eliminate all the pitfalls. Kirby et al¹¹ described a single disc method using high concentration discs. The high potency disc method requires considerable zone measuring. In our experience high concentration discs are more inclined to indicate a greater degree of in vitro susceptibility than is actually present. On the other hand the use of low concentration discs maintains susceptibility in a lower area and we feel is nearer the true susceptibility, in vitro, of the species being tested. If an organism by the low disc method is identified as "moderately resistant" and if the organism is really a "susceptible" species, the administration by the physician of a larger dose of drug compatible with the "moderately resistant" report would not in essence be wrong. The remaining question concerns the interpretation of the report by the physician. An accepted definition of "susceptible," "moderately resistant," and "resistant" would be most helpful in interpreting results. To what do the terms apply?

It would seem that a proper definition of "susceptible" should be related to the in vivo level of drug to be expected with the usual dosage of antimicrobial referable to the route of administration. A "moderately resistant" organism would then be one inhibited at the upper limit of the in vivo level to be expected with the usually prescribed dosage referable to the route of administration. The physician interpreting a "moderately resistant" report would be alerted to use a larger dose of the drug providing no other drug were acceptable. "Resistance" implies an in vitro susceptibility beyond an in vivo level to be expected regardless of dose or route of administration. Since the part played by the body in conjunction with antimicrobial therapy cannot be measured, it must be recognized that definitions apply only in a broad sense. However, there appear to be few, if any, reports of successful antimicrobial therapy with organisms identified as resistant to the in vitro tests. It is more evident that therapy fails in the face of in vitro reports of susceptibility. Until the value to be assessed to the in vivo role is known exactly, it seems advisable that efforts should be directed toward acceptance and approval of a workable practical standard method of in vitro testing. Helpful information relative to interpretation of the test results should be transmitted to the physician perhaps through a protocol similar to that used in Sweden.²⁵

The move by the Food and Drug Administration to certify²³ commercial dehydrated discs is an excellent step forward and is a basis around which a standard disc method can be developed.

The proposed use of two disc concentrations and interpreting results based on any zone around each disc to identify susceptible species is not, in our opinion, suitable as a standard method with the present tentative suggested disc concentrations. Organisms that are "moderately resistant" will tend to give zones with both discs, and organisms that could be "resistant" will present some zone with only the higher concentration. It is probable that if the tentative disc potencies now suggested were lowered the two disc method, considering any zone at all susceptible, would be satisfactory. There is, however, another criticism and resistance to using two discs routinely. The method takes more time, more media, more discs, and is therefore more costly.

It would be preferable in our opinion to use a single low contraction disc, perhaps lower in concentration than is now available, which would permit an interpretation simply by the presence or absence of a zone for susceptible organisms. A higher concentration disc could then be used in a follow-up test; if the primary test results were all negative this disc would then identify moderate resistance.

A definite need for agreement must be established referable to terminology, methodology, and interpretation if the disc testing program is going to fulfill its intent as a helpful therapeutic guide for the patient and the physician.²⁶

SUMMARY

1. The need for a standard method of disc testing is evident from the results of recent hospital laboratory surveys.
2. A procedure for susceptibility testing using low potency commercial dehydrated discs has been presented.
3. Certain problems of disc testing have been pointed out.
4. Variables often referred to in disc testing are not in our opinion major sources of error in the test as it is used in the routine diagnostic laboratory.

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Initial Comparative Study of Susceptibility Tests as Performed with a Replicating Device

ELWOOD L. FOLTZ, EDWARD STEERS, BETTY S. GRAVES,
AND H. JOAN SURIANO

*Section of Infectious Diseases and the William Pepper Laboratory of Clinical Medicine,
School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

In general, two types of sensitivity tests have come into popular use in most hospital laboratories. The easier and perhaps less reliable method of sensitivity testing—the use of commercially available impregnated discs—has gained wide usage because of the ease with which the test could be performed; however, the method is open to many criticisms because of the inconsistencies noted due to variations in content of the antibiotic substance in the discs, to the rate at which diffusion of these substances occurs from the disc to the agar plate, and to the variations in interpretation of the responses obtained with this method. The more satisfactory and perhaps more critical method, which employs a tube dilution of the antibiotic with a constant inoculum, is time consuming to prepare and requires considerable equipment and materials.

The method that had been employed in this laboratory for many years was based on a plate dilution test in which the antibiotic was incorporated in a suitable blood agar medium. Because of the increasing number of antimicrobials over the years and because of the increasing frequency with which sensitivity tests were used for guidance of therapy, a large amount of time was expended in the performance of this test. It was no longer practical to perform serial dilution tests on each organism, and few physicians were sufficiently informed to interpret the significance of such results. A practical solution to these problems was found in performing the sensitivity test at a single concentration of the antibiotic in the blood agar plate. This concentration was chosen with reference to the average cumulative concentrations of each antibiotic in the serum of individuals on full therapeutic dosage regimens. The method was still somewhat cumbersome, due to the time required for inoculation.

In a recent publication¹ from this laboratory, an apparatus for replicate inoculation of an antibiotic containing blood plate medium was described. The device permitted inoculation of the medium with a maximum of 36 individual organisms at one time for the purpose of testing susceptibility to the antibiotic contained in the medium.

It is the purpose of this paper to compare the results obtained with this inoculating device with results from similar antibiotic-containing plates inoculated by the loop-streak method; further comparisons with tube dilution and medicated disc techniques at a single concentration are included in this report.

This study was supported in part by a grant-in-aid from The Upjohn Company, Kalamazoo, Mich.

Organisms. The organisms used throughout this study were speciated pure cultures isolated from clinical materials submitted for bacteriological analysis to the Bacteriology Laboratory, Hospital of the University of Pennsylvania.

Media. Brain-heart infusion broth (Difco) was used to culture the inocula for all tests. This medium was employed in the tube dilution method. The basal plate culture medium consisted of Trypticase soy agar (BBL) supplemented with 1 per cent proteose peptone no. 3 (Difco) and 5 per cent human blood. This medium, without the addition of antibiotics, was used for the paper disc method and as a growth control medium for the plate dilution method. Antibiotic-containing medium was prepared for use in the latter method by adding a specific quantity of the individual antibiotic to the medium.

Antibiotics. Antibiotic standard stock solutions used to prepare the culture media for the tube and plate dilution tests were made from standard reference materials of known potency supplied by the various manufacturers of the individual antibiotics. The standard stock solutions were prepared monthly by dissolving a weighed amount of antibiotic in sterile diluent. Multiple aliquots of each antibiotic standard solution were then frozen until required for the preparation of antibiotic-containing medium. Plate and tube dilution media were prepared twice weekly. The former media was stored at 6 C. and the latter media frozen at -20°C . until used. The concentration of antibiotic per unit volume of media was the same for both the plate and the tube dilution media; concentrations for each antibiotic were based on average blood concentrations attainable with the average dosage regimen.

The antibiotic-containing paper discs (BBL) were purchased from commercial sources. A single disc with a reported specific antibiotic content was employed for each antibiotic tested. The choice of antibiotic content per disc was based largely on a content two times greater or equal to the concentration of the antibiotic in the tube dilution or agar plate. The content per disc for each antibiotic is given in the appropriate tables as the first of two figures enclosed in parentheses. The second figure is the concentration of antibiotic per ml. of blood plate medium.

Inocula. With the exception of the tube dilution tests, the inocula for all other tests were taken from the beginning logarithm phase of division and consisted of a suspension of 10^7 cells per ml. of brain-heart infusion. The inocula for the tube dilution tests were prepared by combining 1 ml. of a 1:10,000 suspension of cells from a 16 hour brain-heart infusion culture with 1 ml. of brain-heart infusion containing the antibiotic, and thus achieving the final specific concentration.

Plate Dilution Method. Two methods of inoculation were used. The first was the replica inoculation method, which has been described in detail in a previous report.¹ By this method a Trypticase soy agar-blood control plate or an antibiotic-containing Trypticase soy agar-blood plate may be inoculated with a maximum of 36 cultures by a single manipulation of the inoculating mechanism. In the second, the loop-streak method of inoculation was performed with the same source of inocula used in the replica inoculation method. In this method the Trypticase soy agar-blood medium was streaked with the inoculum contained by a 1.5 mm. platinum loop. Plates inoculated by both methods were incubated at 37°C . for 16 to 18 hours. The presence of one or more colonies on an antibiotic-containing plate was interpreted as evidence of resistance to the antibiotic.

TABLE I

Per Cent Agreement of Results of Sensitivity Tests by Loop-Streak and Replicate Inoculation

Organism	No. tests	Penicillin		Streptomycin	Tetracycline	Chloramphenicol
		1 u./ml.	5 u./ml.	15 µg./ml.	5 µg./ml.	15 µg./ml.
Staphylococci	196	90	83	90	94	98
Streptococci						
Alpha-hemolytic	6	100	100	100	100	100
Beta-hemolytic	12	100	91	100	100	100
Gamma-hemolytic	29	97	90	100	97	90
<i>E. coli</i>	57	100	96	90	90	96
<i>Pseudomonas</i>	35	100	100	97	100	100
<i>Aerobacter-Klebsiella</i>	20	100	100	100	95	100
Paracolon	1	100	100	100	100	100
<i>Alcaligenes</i>	6	100	100	100	100	100

Tube Dilution Method. A single tube of brain-heart infusion broth containing a specific concentration of antibiotic was inoculated with a culture as described in the section "Inocula." The cultures were incubated for 16 to 18 hours at 37 C. Visible turbidity due to bacterial multiplication was considered evidence for resistance.

Paper Disc Method. The Trypticase soy agar-blood medium was inoculated by streaking the surface with a sterile swab moistened with the culture. After drying for 15 to 20 minutes, the antibiotic discs were dropped onto the surface of the agar from a mechanical dispenser (BBL). The inverted plates were incubated at 37 C. for 16 to 18 hours. It was arbitrarily decided that an inhibitory zone of 12 mm. or more constituted evidence for sensitivity to the antibiotic. Conversely, a zone of less than 12 mm. was interpreted as evidence for resistance.

TABLE II

*Comparison of Replicate Inoculation and Loop-Streak Agar Plate Sensitivity Tests**

Data	Penicillin		Strepto- mycin	Tetra- cycline	Chloram- phenicol	Erythro- mycin	Novo- biocin
	1 u./ml.	5 u./ml.	15 µg./ml.	5 µg./ml.	15 µg./ml.	4 µg./ml.	25 µg./ml.
Staphylococci							
Per cent agreement	90	83	90	94	98	97	96
No. tested	196	196	196	196	196	193	193
SS	54	67	94	119	160	147	174
RR	123	96	83	65	33	41	12
SR	18	33	19	12	2	5	6
RS	1				1		1
<i>E. coli</i>							
Per cent agreement	100	96	90	90	96	(not tested)	
No. tested	57	57	57	57	57		
SS	0	0	34	34	42		
RR	57	55	17	17	13		
SR		2	5	5	2		
RS			1	1			

* SS = sensitive by both methods; RR = resistant by both methods; SR = sensitive by loop, resistant by replicator; RS = resistant by loop, sensitive by replicator.

TABLE III

Agreement of Replicate Inoculation Plate and Tube-Dilution Sensitivity Tests

	Oleandomycin		Bacitracin		Vancomycin		Ristocetin		Kanamycin		Neomycin		Polymyxin	
	No.	Agree- ment	No.	Agree- ment	No.	Agree- ment	No.	Agree- ment	No.	Agree- ment	No.	Agree- ment	No.	Agree- ment
Staphylococci	371	67	371	73	367	74	359	90	382	67	381	93	NT	
Streptococci														
Alpha-hemolytic	16	69	16	75	16	81	17	70	17	82	18	72	NT	
Beta-hemolytic	13	54	13	69	12	58	13	54	12	67	13	54	NT	
Gamma-hemolytic	23	39	23	91	21	67	23	52	26	81	23	83	NT	
<i>E. coli</i>	NT*		NT		NT		NT		136	65	136	81	134	63
<i>Pseudomonas</i>	NT		NT		NT		NT		97	93	95	61	95	64
<i>Proteus</i>	NT		NT		NT		NT		69	71	68	59	66	98
<i>Aerobacter</i>	NT		NT		NT		NT		52	85	52	75	50	64
<i>Alcaligenes</i>	NT		NT		NT		NT		16	87	17	88	16	75

* NT = not tested.

RESULTS

The results of the various comparisons are shown in the accompanying tables I to VII.

Table I contains results that indicate remarkable agreement between replicate inoculation and loop-streak inoculation of antibiotic-containing blood agar plates. The agreement in results for the two methods for 362 different isolates belonging to seven major genera of organisms was generally 90 per cent or better. The greatest variation between the two methods occurred with the staphylococci and *Escherichia coli*.

As noted in table II, staphylococci were more susceptible when inoculated by the loop-streak method on plates containing penicillin, streptomycin, and tetracycline.

In 1366 staphylococcal susceptibility trials to various antibiotics, susceptibility

TABLE IV

*Comparison of Replicate Inoculation Plate and Tube-Dilution Sensitivity Tests**

Data	Oleando- mycin 2 µg./ml.	Baci- tracin 1 u./ml.	Vanco- mycin 5 µg./ml.	Risto- cetin 5 µg./ml.	Kana- mycin 15 µg./ml.	Neo- mycin 30 µg./ml.
Staphylococci						
Per cent agreement	67	73	74	90	67	93
No. tested	371	371	367	359	382	381
SS	174	28	254	1	220	307
RR	76	244	17	324	35	47
SR	88	64	49	27	79	18
RS	33	35	47	7	48	7
Streptococci (gamma-hemolytic)						
Per cent agreement	39	91	67	52	81	83
No. tested	23	23	21	23	26	23
SS	3	2	13	8	0	0
RR	6	19	1	4	21	19
SR	14	1	6	8	0	3
RS	0	1	1	3	5	1

* SS = sensitive by both methods; RR = resistant by both methods; SR = sensitive in tube, resistant by replicator; RS = resistant in tube, sensitive by replicator.

TABLE V

Comparison of Replicate Inoculation Plate and Tube Dilution Sensitivity Tests*

Data	Kana- mycin, 15 µg./ml.	Neo- mycin, 30 µg./ml.	Poly- myxin, 25 µg./ml.	Combination†		
				A	B	C
<i>E. coli</i>						
Per cent agreement	65	81	63	78	75	74
No. tested	136	136	134	135	135	134
SS	56	106	68	63	78	72
RR	32	4	16	43	24	27
SR	20	7	27	10	17	6
RS	28	19	23	19	16	29

* SS = sensitive by both methods; RR = resistant by both methods; SR = sensitive in tube, resistant by replicator; RS = resistant in tube, sensitive by replicator.

† A = penicillin, 1 u./ml., plus streptomycin, 15 µg./ml.; B = penicillin, 1 u./ml., plus chloramphenicol, 15 µg./ml.; C = streptomycin, 15 µg./ml., plus tetracycline, 5 µg./ml.

was noted following inoculation by the replicator in only three instances when resistance was noted in the loop-streaked plates at the same time. On the other hand, in 95 of these trials, susceptibility was noted on the loop-streak plates with resistance noted for the replicate inoculator. Strains of *E. coli* were similarly more susceptible to streptomycin and tetracycline following loop-streak inoculation. Fourteen of 285 tests with five different antimicrobials favored susceptibility of *E. coli* when inoculated by the loop-streak method and resistance to inoculation with the replicator; two strains were sensitive by the replicate inoculation when resistant to the loop-streak inoculations.

The comparison of the replicate inoculation plate dilution results with those of the tube dilution tests were limited to those antibiotics of relatively recent origin or for which sensitivity tests were not routinely performed in this laboratory. For the gram-positive organisms, tests were carried out with oleandomycin, bacitracin, vancomycin, ristocetin, kanamycin, and neomycin. For the gram-negative organisms, the studies were carried out primarily with kanamycin, neomycin, and polymyxin; three empiric combinations were also employed for the gram-negative organisms. From table III, it is obvious that the agreement between the two methods was generally 70 per cent or better.

The variations between the two methods are best demonstrated for gram-positive

TABLE VI

Per Cent Agreement of Results of Sensitivity Tests by Commercial Disc and Replicate Inoculation Methods Against Gram-Positive Organisms*

No. tests	Penicillin (2-1)	Erythro- mycin, (10-5)	Erythro- mycin, (15-4)	Novo- biocin, (30-25)	Strepto- mycin, (10-15)	Tetra- cycline, (5-5)	Chloram- phenicol, (30-15)	Baci- tracin, (2-1)	Vanco- mycin, (30-5)	Risto- cetin, (30-5)	Kana- mycin, (30-15)	Neo- mycin, (30-15)
Staphylococci												
261	85	84	87	89	94	96	90	49	84	38	76	87
Streptococci												
75	61	90	96	77	85	87	80	76	95	80	53	70

* The first number in parentheses expresses antibiotic content of disc; the second number expresses the concentration of antibiotic in µg./ml. of medium.

TABLE VII

Agreement of Results of Sensitivity Tests by Commercial Disc and Replicate Inoculation Methods Against Gram-Negative Organisms*

Organism	No. tests	Streptomycin (10-15)	Tetracycline (5-5)	Chloramphenicol (30-15)	Kanamycin (30-15)	Neomycin (30-30)
<i>E. coli</i>	139	92	73	92	73	95
<i>Pseudomonas</i>	83	86	95	69	73	79
<i>Aerobacter-Klebsiella</i>	82	96	80	90	91	93
<i>Proteus</i>	70	77	99	80	49	56

* The first number in parentheses expresses antibiotic content of disc; the second number expresses the concentration of antibiotic in $\mu\text{g./ml.}$ of medium.

organisms in table IV. The number of organisms susceptible to the tube dilution method and resistant to the replicate inoculation plate generally exceeded the number sensitive to the replicator but resistant to the tube method. The most notable exception to this trend occurred with kanamycin, which appeared to show resistance to the tube dilution method with streptococci but susceptibility when tested on the replicate inoculation plate. The results of the tests with *E. coli* are similarly shown in table V, in which the difference in response to the two methods is tabulated. Testing various strains of *E. coli* with kanamycin and neomycin showed a higher proportion of organisms susceptible by the replicator test than by the tube method when disagreement existed. This is also true of the empirical combinations of penicillin with streptomycin and streptomycin with tetracycline. The agreement between the two methods was quite good for neomycin but decidedly less for kanamycin and polymyxin.

The agreement noted between results for susceptibility tests as performed with the replicator plate and the commercial disc is recorded in tables VI and VII. Agreement for the gram-positive isolates tested is relatively high for all antibiotics, with the exception of ristocetin, bacitracin, and kanamycin. The gram-negative isolates exceeded 70 per cent agreement with all tests, except for *Proteus* species to kanamycin and for *Pseudomonas* species to chloramphenicol.

DISCUSSION

The contribution to methodology of sensitivity testing reported here is that of a rapid inoculation of blood agar plates containing antibiotic. The employment of antibiotic-containing plates has been established as a useful method by others.^{2,4} The laborious hand streaking by loop of each organism to be tested to each antibiotic is thus eliminated. The need for comparison of results obtained by replicate inoculation plates with those of various other methods is obvious.

The excellent agreement of results by the replicator and hand-streaked plates at a single concentration of antibiotic was anticipated. The only major differences between the methods were the inoculum size and distribution of inoculum. With the loop, approximately 0.0005 ml. of the broth culture is streaked in a small segment of the plate; with the replicator, approximately 0.005 ml. of culture is deposited as a droplet on the surface with little dispersion. With the routine use of a single concentration of the commonly employed antibiotics as a screening sensitivity test, the

results were sufficiently comparable to have confidence in the new method of inoculation.

When disagreement between results of the two methods occurred, the susceptibility was noted 95 times for the loop method as compared to three instances for the replicate inoculation. This suggests that the disagreement may be related to inoculum size, which, in the loop-streak method, is roughly 5000 cells and, with the replicator, approximately 50,000; the larger inoculum was confined to a smaller surface area, and thus resistance may have been favored by the ratio of organisms to the available antibiotic.

The comparison of the tube dilution method with the replicator-inoculated antibiotic plate introduced variations of media and of the environment for the growing organisms. The concentrations of the individual antibiotics in the two media were kept similar. The inocula were equal in numbers of organisms but were different in relation to volume of media and the available antibiotic. In spite of these basic differences in performance of the tests, the agreement was generally satisfactory, and about 70 per cent or better of isolates tested showed agreement between the two methods of sensitivity testing.

The agreement was poor between tube and plate for certain drugs with the non-hemolytic and beta-hemolytic streptococci tested. This study was performed on a limited number of these organisms, which were not typed for groups but simply differentiated on the characteristic of hemolysis. We recognize certain factors to be of probable significance in accounting for the variation but have no direct evidence to support these hypotheses. Among these factors may be the differences in media and the gas tensions in the environment of the organisms in the two tests. The inocula, although of approximately equal numbers, were from cultures of different age and, therefore, of different growth activity; this may also influence the susceptibility to these antibiotics.

The agreement noted between the commercial disc method and the replicator is generally good by employing certain criteria of zone size for inhibition of organisms by the disc. This agreement is discussed more completely in the following publication.⁵ The poorest agreement was noted with staphylococci to bacitracin and ristocetin.

The replicate inoculation of antibiotic-containing blood agar plates appears to be a satisfactory method of determining bacterial sensitivity. It speeds the operation of inoculation and gives results comparable with similar tests performed with loop-streaked plates in the plate dilution method. Thus, a major objection to the use of plate dilution techniques in bacterial sensitivity tests has been eliminated. The other inherent advantages of more precise quantitation of the drug concentration at which the test is run and the limited end point, as described, permit a simple and definitive evaluation of antibiotic effectiveness.

SUMMARY

Preliminary results of sensitivity tests with a replicate inoculation of blood agar plates, containing a single concentration for each of the antibiotics tested, showed excellent agreement when compared to loop-streak inoculation of similar plates.

Similar comparison of the replicator results with those of tube dilution and com-

mercial disc sensitivity methods show satisfactory agreement, in spite of certain obvious differences in methods.

These data suggest the usefulness of the method as a satisfactory in vitro screening method for antibiotic inhibition of bacterial pathogens. Its chief advantages are in the speed of performance, the precision of antibiotic concentration to which the organism is exposed, and the limited end point for interpretation of results.

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Comparison of Bacterial Susceptibility to Antibiotics as Determined by the Plate Dilution Method and by the Disc Method

EDWARD STEERS, ELWOOD L. FOLTZ, BETTY S. GRAVES,
AND H. JOAN SURIANO

*Section of Infectious Diseases and the William Pepper Laboratory of Clinical Medicine,
School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

Information derived from antibiotic sensitivity testing is useful for determining the selection of appropriate agents for therapy of bacterial infections. The increasing and widespread use of a growing number of antibiotics, with a concomitant emergence of drug-resistant strains of certain species, has emphasized the need for a reliable qualitative antibiotic sensitivity test.

The tube and plate dilution methods yield reasonably accurate semiquantitative results. However, they have been too unwieldy for use in performing routine clinical testing in the average laboratory. Thus, for the most part, their use has been limited to special situations in which the need for a semiquantitative result justifies employing these methods.

The paper disc method is used most frequently to obtain qualitative results in routine sensitivity testing. However, the variation in performance of the test, the lack of production quality control, attempts to semiquantitate the method, etc., have led to considerable confusion between reports in the literature.

The plate dilution method is used in our laboratory because it eliminates the objections to the paper disc method by providing an accurately known distribution of the antibiotic in the medium and an easier result to interpret on the basis of any bacterial growth in the presence of the antibiotic. A recent report¹ from this laboratory, describing an inocula replicating apparatus for use with the plate dilution method, has answered the objection to the method's unwieldiness for routine sensitivity testing.

This report deals with a comparison of the antibiotic sensitivity results obtained by the plate dilution method, utilizing the replica inoculating apparatus, and the paper disc method.

MATERIALS AND METHODS

The preceding report² describes in detail the source of the organisms, media, inocula, methodology, etc., used in that comparative study and the one reported here. Though the same species of organisms were used for both studies, the specific cultures used for each were not identical. However, the same cultures and inocula were used in this comparative study of the sensitivity results as determined by the paper disc and plate dilution methods.

This study was supported in part by a grant-in-aid from The Upjohn Company, Kalamazoo, Mich.

TABLE I

*Comparison of the Sensitivity of Staphylococci to Antibiotics by the Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.	RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement								
Chloramphenicol, (30-15)†																			Penicillin, (2-1)				Penicillin, (10-5)			
A 6	31		2		33	94	142		2		144	99	118		2		120	98								
8	6		0		6	100	3		0		3	100	4		0		4	100								
10	14		1		15	93	18		1		19	95	11		1		12	92								
Total A Av.	51		3		54	94	163		3		166	98	133		3		136	98								
B 12		1		1	2	50	3		20	23	13		5		17	22	23									
14		1		1	2	50	0		6	6	—		6		11	17	35									
16		1		2	3	33	0		2	2	—		10		5	15	67									
18		8		3	11	73	4		2	6	67		4		1	5	80									
20		44		6	50	88	1		0	1	100		2		2	4	50									
22		56		5	61	92	3		0	3	100		5		0	5	100									
24		52		3	55	95	15		3	18	83		12		0	12	100									
26		22		1	23	96	34		2	36	94		43		2	45	96									
Total B Av.	185		22		207	89	60		35	95	63		87		38	125	70									
Total A plus B Av.	236		25		261	90	223		38	261	85		220		41	261	84									
Streptomycin, (10-15)																			Tetracycline, (5-5)				Erythromycin, (15-4)			
A 6	142		2		144	99	156		3		159	98	63		7		70	90								
8	4		0		4	100	1		0		1	100	6		0		6	100								
10	3		0		3	100	2		4		6	33	6		1		7	86								
Total A Av.	149		2		151	99	159		7		166	96	75		8		83	90								
B 12		2		1	3	67	8		0	8	100		2		2	4	50									
14		23		5	28	82	6		1	7	86		2		3	5	40									
16		20		1	21	95	26		2	28	93		0		6	6	—									
18		14		3	17	82	21		1	22	96		6		5	11	55									
20		19		1	20	95	21		0	21	100		9		1	10	90									
22		9		2	11	82	10		0	10	100		25		1	26	96									
24		6		0	6	100	9		1	10	90		63		5	68	93									
26		3		1	4	75	1		0	1	100		46		3	49	94									
Total B Av.	96		14		110	87	102		5	107	95		153		26	179	86									
Total A plus B Av.	245		16		261	94	261		12	273	96		228		34	262	87									
Novobiocin, (30-25)																			Bacitracin, (2-1)				Vancomycin, (30-5)			
A 6	8		3		11	73	17		5		22	77	6		2		8	75								
8	1		0		1	100	5		0		5	100	0		0		0	—								
10	12		5		17	71	23		2		25	92	0		4		4	—								
Total A Av.	21		8		29	72	45		7		52	87	6		6		12	50								
B 12		12		7	19	63	13		34	47	28		11		1	12	92									
14		11		3	14	79	25		56	81	31		53		16	69	77									
16		18		1	19	95	25		26	51	49		101		15	116	87									
18		46		5	51	90	10		5	15	67		38		2	40	95									
20		52		2	54	96	3		1	4	75		6		0	6	100									
22		25		0	25	100	1		0	1	100		1		0	1	100									
24		30		1	31	97	0		0	0	—		0		1	1	—									
26		6		0	6	100	1		0	1	100		0		0	0	—									
Total B Av.	200		19		219	91	78		122	200	39		210		35	245	86									
Total A plus B Av.	221		27		248	89	123		129	252	49		216		41	257	84									

Table I Continued on Page 606

TABLE I (Continued)

*Comparison of the Sensitivity of Staphylococci to Antibiotics by the
Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.						% agree- ment						% agree- ment						% agree- ment
	RR	SS	RS	SR	Total		RR	SS	RS	SR	Total		RR	SS	RS	SR	Total	
Neomycin, (30-30)																		
A 6	7		1		8	88	4		2		6	67	9		0		9	100
8	0		0		0	—	0		0		0	—	5		0		5	100
10	1		1		2	50	3		0		3	100	80		1		81	99
Total A	8		2		10		7		2		9		94		1		95	
Av.						80					78							99
Kanamycin, (30-15)																		
B 12		6		1	7	86		2		1	3	67		4		133	137	3
14		20		4	24	83		1		6	7	14		0		24	24	—
16		71		10	81	88		18		15	33	55		1		3	4	25
18		51		4	55	93		55		19	74	74		0		0	0	—
20		21		3	24	88		51		12	63	81		1		1	2	50
22		20		7	27	74		15		4	19	79		0		1	1	—
24		15		1	16	94		28		2	30	93		0		1	1	—
26		9		1	10	90		26		2	28	93		0		0	0	—
Total B		213		31	244		196		61	257		76		6		163	169	
Av.						87												4
Total A plus B Av.		221		33	254		203		63	266		76		100		164	264	38
Ristocetin, (30-5)																		

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

DISCUSSION OF RESULTS

Tables I through VI list the sensitivity results for 710 organisms representing six genera. These results were obtained by parallel use of the paper disc and plate dilution methods. All organisms were tested by both methods against the 12 antibiotics listed for the staphylococci in table I. However, the results for an antibiotic have been omitted from the table in those instances in which all strains of a species or genus were uniformly resistant by both methods.

The results for the paper disc method are recorded in the tables in terms of zone diameter of growth inhibition. Twelve mm. was arbitrarily selected as the minimum zone diameter for an organism to be characterized as sensitive to the antibiotic. Conversely, a zone diameter less than 12 mm. was interpreted as resistance. The criterion for sensitivity of an organism to an antibiotic by the plate dilution method was complete inhibition of growth. One or more visible colonies was interpreted as resistance.

Examination of table I reveals that of 261 strains of staphylococci tested for sensitivity to chloramphenicol with the 30 $\mu\text{g.}$ disc, 54 yielded zone diameters of less than 12 mm. and were therefore considered resistant. When these strains were tested by the plate dilution method, 51 strains were resistant to 15 $\mu\text{g./ml.}$ of chloramphenicol and three strains were sensitive. The agreement of the two methods with regard to determining resistance to chloramphenicol at the levels of the antibiotic used was 94 per cent. Furthermore, it is evident from table I that 207 of the strains tested with the 30 $\mu\text{g.}$ paper disc yielded zones of 12 mm. or more in diameter and thus were considered sensitive to chloramphenicol. By the plate dilu-

TABLE II

*Comparison of the Sensitivity of Streptococci to Antibiotics by the Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.						% agreement						% agreement						% agreement										
	RR	SS	RS	SR	Total		RR	SS	RS	SR	Total		RR	SS	RS	SR	Total											
Chloramphenicol, (30-15)†																			Penicillin, (2-1)					Penicillin, (10-5)				
A 6	7		3		10	70	11		1		12	92	2		4		6	33										
8	4		0		4	100	1		0		1	100	0		0		0	—										
10	1		2		3	33	16		0		16	100	0		0		0	—										
Total A	12		5		17		28		1		29		2		4		6											
Av.						71						97						33										
Penicillin, (2-1)																			Penicillin, (10-5)									
B 12		2		0	2	100	1		14		15	7		2		1	3	67										
14		2		0	2	100	0		10		10	—		5		1	6	83										
16		3		1	4	75	0		1		1	—		11		4	15	73										
18		8		4	12	67	0		1		1	—		11		4	15	73										
20		12		3	15	80	1		0		1	100		8		0	8	100										
22		8		0	8	100	0		1		1	—		3		0	3	100										
24		5		1	6	83	5		0		5	100		5		0	5	100										
26		9		2	11	82	11		1		12	92		14		0	14	100										
Total B		49		11	60		18		28		46			59		10	69											
Av.						82						39						86										
Total A plus B		61		16	77		46		29		75			67		14	75											
Av.						80						61						81										
Streptomycin, (10-15)																			Tetracycline, (5-5)					Erythromycin, (15-4)				
A 6	56		1		57	98	41		2		43	95	3		2		5	60										
8	1		0		1	100	1		0		1	100	0		0		0	—										
10	2		1		3	67	0		1		1	—	1		0		1	100										
Total A	59		2		61		42		3		45		4		2		6											
Av.						97						93						67										
B 12		0		2	2	—	5		2		7	71		2		0	2	100										
14		0		2	2	—	2		1		3	66		0		0	0	—										
16		0		1	1	—	6		1		7	85		2		0	2	100										
18		0		0	0	—	2		1		3	66		4		1	5	80										
20		0		1	1	—	2		0		2	100		14		0	14	100										
22		1		0	1	100	2		0		2	100		21		0	21	100										
24		2		1	3	67	2		0		2	100		8		0	8	100										
26		2		2	4	50	2		2		4	50		15		0	15	100										
Total B		5		9	14		23		7		30			66		1	67											
Av.						36						77						99										
Total A plus B		64		11	75		65		10		75			70		3	73											
Av.						85						87						96										
Novobiocin, (30-25)																			Bacitracin, (2-1)					Vancomycin, (30-5)				
A 6	3		2		5	60	9		2		11	82	3		1		4	75										
8	0		1		1	—	0		0		0	—	0		0		0	—										
10	1		2		3	33	17		0		17	100	0		0		0	—										
Total A	4		5		9		26		2		28		3		1		4											
Av.						44						93						75										
Bacitracin, (2-1)																			Vancomycin, (30-5)									
B 12		10		6	16	63	4		13		17	24		1		1	2	50										
14		14		5	19	74	7		2		9	78		10		0	10	100										
16		13		1	14	93	6		1		7	86		28		2	30	93										
18		6		0	6	100	5		0		5	100		13		0	13	100										
20		4		0	4	100	0		0		0	—		4		0	4	100										
22		0		0	0	—	1		0		1	100		3		0	3	100										
24		2		0	2	100	1		0		1	100		4		0	4	100										
26		5		0	5	100	6		0		6	100		3		0	3	100										
Total B		54		12	66		30		16		46			66		3	69											
Av.						82						65						95										
Total A plus B		58		17	75		56		18		74			69		4	73											
Av.						77						76						95										

Table II Continued on Page 608

TABLE II (Continued)

Comparison of the Sensitivity of *Streptococci* to Antibiotics by the Disc Method and the Pour Plate Dilution Method*

Zone diameter, mm.		RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement
Ristocetin, (30-5)							Kanamycin, (30-15)							Neomycin, (30-30)					
A	6	4		1		5	80	33		3		36	92	35		2		37	95
	8	0		0		0	—	2		0		2	100	2		0		2	100
	10	1		0		1	100	4		0		4	100	5		4		9	56
Total A		5		1		6		39		3		42		42		6		48	
Av.							83						93						88
B	12		9		4	13	69	0		6	6	—		3		4	7	43	
	14		23		9	32	73	0		14	14	—		5		6	11	46	
	16		11		2	13	85	0		6	6	—		1		5	6	17	
	18		7		0	7	100	0		3	3	—		2		0	2	100	
	20		3		0	3	100	1		1	2	50		0		2	2	—	
	22		1		0	1	100	0		0	0	—		0		0	0	—	
	24		1		0	1	100	0		0	0	—		1		0	1	100	
	26		1		0	1	100	1		3	4	25		0		0	0	—	
Total B			56		15	71		2		33	35			12		17	29		
Av.							79						6						41
Total A plus B			61		16	77		41		36	77			54		23	77		
Av.							79						53						70

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

tion method, 185 strains were sensitive to 15 $\mu\text{g./ml.}$ of chloramphenicol and 22 were resistant. Thus, the agreement of the two methods with regard to determining sensitivity to chloramphenicol was 89 per cent. The agreement between the two methods for both resistance and sensitivity to chloramphenicol was based on 236 of 261 strains of staphylococci, or 90 per cent.

It is evident from a similar analysis of the data in table I that, with the exception of bacitracin and ristocetin, the over-all agreement between the two methods for each antibiotic was 76 per cent or better, with an average agreement of 87 per cent.

The poor agreement between the two methods in the case of ristocetin may be explained by the fact that the ratio of the antibiotic content in the disc to that per ml. of medium is 6:1. Because small zones were anticipated with ristocetin discs, the high content discs (30 $\mu\text{g.}$) were used to insure the appearance of measurable zones for organisms sensitive to this antibiotic. It is of interest to note that though the disc content:plate concentration ratio was the same (6:1) for vancomycin, the agreement was 84 per cent. The lack of good agreement for the tests involving bacitracin is not as readily explained.

From table II it can be seen that for streptococci the variation in agreement between the two methods for the antibiotics listed was from 53 to 96 per cent, with an average agreement of 78 per cent.

Examination of tables III to VI, which list the sensitivity results for the gram-negative organisms, will reveal that: for *Escherichia coli* (table III), the variation in agreement was from 73 to 95 per cent, with an average agreement of 85 per cent; for *Aerobacter aerogenes* (table IV), the variation was from 79 to 96 per cent,

with an average agreement of 90 per cent; for *Proteus* species (table V), the variation was from 49 to 99 per cent, with poor agreements for kanamycin and neomycin at 49 and 56 per cent, respectively, the average agreement being 73 per cent; and finally, for *Pseudomonas* species (table VI), the variation was from 45 to 95 per cent, with an average agreement of 75 per cent. It is interesting that for polymyxin B, one of the most useful antibiotics for therapy of infections due to *Pseudomonas* species, the agreement between the two methods was 45 per cent. The disc content for this antibiotic was 50 units, whereas the concentration of polymyxin B per ml. of plate medium was 25 units. In spite of this, the lack of good agreement results from the fact that while 57 of the 82 strains tested were resistant by the disc method, 40

TABLE III
*Comparison of the Sensitivity of E. coli to Antibiotics by the
Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.	RR	SS	RS	SR	Total	% agree- ment	RR	SS	RS	SR	Total	% agree- ment	RR	SS	RS	SR	Total	% agree- ment
Chloramphenicol, (30-15)†							Streptomycin, (10-15)						Tetracycline, (5-5)					
A 6	35		1		36	97	54		1		55	98	60		19		79	76
8	1		0		1	100	1		1		2	50	2		3		5	40
10	1		0		1	100	2		1		3	67	5		12		17	29
Total A Av.	37		1		38	97	57		3		60	95	67		34		101	66
B 12		0		1	1	—	16		2		18	89	13		3		16	81
14		0		2	2	—	26		3		29	90	16		0		16	100
16		3		2	5	60	20		3		23	87	4		0		4	100
18		16		3	19	84	6		0		6	100	2		0		2	100
20		27		1	28	96	2		0		2	100	0		0		0	—
22		22		0	22	100	0		0		0	—	0		0		0	—
24		22		0	22	100	1		0		1	100	0		0		0	—
26		0		1	1	—	0		0		0	—	0		0		0	—
Total B Av.		90		10	100	90	71		8		79	90	35		3		38	92
Total A plus B Av.		127		11	138	92	128		11		139	92	102		37		139	73
Kanamycin, (30-15)							Neomycin, (30-30)											
A 6	4		0		4	100	2		1		3	67						
8	1		0		1	100	0		1		1	—						
10	1		0		1	100	1		2		3	33						
Total A Av.	6		0		6	100	3		4		7	43						
B 12		3		1	4	75	10		0		10	100						
14		5		3	8	63	48		3		51	94						
16		27		11	38	71	49		0		49	100						
18		32		16	48	67	16		0		16	100						
20		20		6	26	77	4		0		4	100						
22		6		0	6	100	0		0		0	—						
24		3		0	3	100	2		0		2	100						
26		0		0	0	—	0		0		0	—						
Total B Av.		96		37	133	72	129		3		132	98						
Total A plus B Av.		102		37	139	73	132		7		139	95						

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

TABLE IV

*Comparison of the Sensitivity of Aerobacter to Antibiotics by the Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.	RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement	RR	SS	RS	SR	Total	% agreement								
Chloramphenicol, (30-15)†																			Streptomycin, (10-5)				Tetracycline , (5-5)			
A 6	38		0		38	100	47		0		47	100	45		9		54	83								
8	0		0		0	—	1		0		1	100	1		0		1	100								
10	1		0		1	100	1		1		2	50	4		3		7	57								
Total A	39		0		39		49		1		50		50		12		12									
Av.						100						98						81								
B 12		1		1	2	50		7		1	8	88		8		3	11	73								
14		0		0	0	—		13		0	13	100		4		2	6	67								
16		0		3	3	—		8		1	9	89		2		0	2	100								
18		3		0	3	100		1		0	1	100		0		0	0	—								
20		8		3	11	73		0		0	0	—		1		0	1	100								
22		7		1	8	88		1		0	1	100		0		0	0	—								
24		9		0	9	100		0		0	0	—		0		0	0	—								
26		6		0	6	100		0		0	0	—		0		0	0	—								
Total B		34		8	42			30		2	32			15		5	20									
Av.						81						94						75								
Total A plus B																										
Av.		73		8	81			79		3	82			65		17	82	79								
Kanamycin, (30-15)																			Neomycin, (30-30)							
A 6	2		0		2	100	0		0		0	—														
8	0		0		0	—	0		1		1	—														
10	1		0		1	100	1		0		1	100														
Total A	3		0		3		1		1		2															
Av.						100						50														
B 12		2		0	2	100		7		3	10	70														
14		7		0	7	100		31		2	33	94														
16		29		2	31	94		32		1	33	97														
18		23		4	27	85		2		0	2	100														
20		11		0	11	100		1		0	1	100														
22		1		0	1	100		0		0	0	—														
24		1		0	1	100		0		0	0	—														
26		0		0	0	—		0		0	0	—														
Total B		74		6	80			73		6	79															
Av.						93						93														
Total A plus B																										
Av.		77		6	83			75		7	82							91								

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

of these strains were sensitive by the plate dilution method. If the criterion for sensitivity for the paper disc method were a zone diameter of 10 rather than 12 mm. the agreement would have been 70 rather than 46 per cent.

Antibiotic solubility and diffusibility influence the size of the inhibitory zones produced, but these properties may not necessarily coincide with antibacterial activity. Thus, the recommendation is made that zone size be ignored in interpreting sensitivity results obtained with the paper disc method. However, Kirby and co-workers³ have reported excellent correlations between the use of a single high content disc and tube dilution tests, as well as clinical results.

All the discs used in this study for the various antibiotics were the high content discs, with the exception of those for streptomycin, tetracycline, bacitracin, and

polymyxin B. Examination of the data in the tables with the purpose of determining, for each antibiotic, which zone size would be the maximum above which the greatest number of sensitive cultures are associated, should aid in establishing for each antibiotic the minimum zone size for characterizing each culture as sensitive. From this viewpoint, it would appear that for chloramphenicol the minimum zone size would be 18 mm. (see tables I to III). With 12 mm. as the minimum zone size for chloramphenicol, the agreements between the two methods for staphylococci, streptococci, and *E. coli* are 90, 80, and 92 per cent, respectively. If 18 mm. were used as the minimum zone size for this antibiotic and these organisms, the agreements would be 91, 71, and 93 per cent, respectively. The same type of

TABLE V
*Comparison of the Sensitivity of Proteus to Antibiotics by the
Disc Method and the Pour Plate Dilution Method**

Zone diameter, mm.						% agree- ment						% agree- ment						% agree- men
	RR	SS	RS	SR	Total		RR	SS	RS	SR	Total		RR	SS	RS	SR	Total	
Chloramphenicol, (30-15)†																		
A	6	24		2	26	92	39		1		40	98	2		3		5	40
	8	0		0	0	—	0		0		0	—	0		0		0	—
	10	1		0	1	100	0		0		0	—	1		0		1	100
Total A		25		2	27		39		1		40		3		3		6	
Av.						92						98						50
B	12		0		0	—		1		4	5	20		0		0	0	—
	14		2		2	50		3		3	6	50		1		3	4	25
	16		4		4	50		7		7	14	50		6		6	12	50
	18		9		4	69		2		1	3	67		11		19	30	4
	20		10		1	91		2		0	2	100		11		3	14	79
	22		5		0	5		0		0	0	—		1		1	2	50
	24		0		0	—		0		0	0	—		1		1	2	50
	26		0		1	1		0		0	0	—		0		0	0	—
Total B			30		12	42		15		15	30			31		33	64	
Av.						71						50						49
Total A plus B Av.			55		14	69				54	16	70			34		36	70
						80						77						49
Neomycin, (30-30)																		
Tetracycline, (5-5)																		
A	6	2		1	3	67	68		0		68	100						
	8	0		0	0	—	0		0		0	—						
	10	1		0	1	100	0		0		0	—						
Total A		3		1	4		68		0		68							
Av.						75						100						
B	12		2		8	10		0		0	0	—						
	14		11		12	23		0		0	0	—						
	16		18		8	26		0		0	0	—						
	18		5		2	7		0		1	1	—						
	20		0		0	0		0		0	0	—						
	22		0		0	0		0		0	0	—						
	24		0		0	0		0		0	0	—						
	26		0		0	0		1		0	1	100						
Total B			36		30	66		1		1	2							
Av.						55						50						
Total A plus B Av.			39		31	70				69	1	70						99
						56						99						

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

TABLE VI

Comparison of the Sensitivity of *Pseudomonas* to Antibiotics by the
Disc Method and the Pour Plate Dilution Method*

Zone diameter, mm.						% agree- ment						% agree- ment						% agree- ment										
	RR	SS	RS	SR	Total		RR	SS	RS	SR	Total		RR	SS	RS	SR	Total											
Chloramphenicol, (30-15)†																			Streptomycin, (10-15)					Kanamycin, (30-15)				
A 6	45		1		46	98	52		0		52	100	32		5		37	87										
8	2		0		2	100	8		0		8	100	8		3		11	77										
10	10		0		10	100	8		3		11	73	12		3		15	80										
Total A Av.	57		1		58	98	68		3		71	96	52		11		63	83										
B 12		0		6	6	—		1		6	7	14		2		8	10	20										
14		0		10	10	—		1		2	3	33		1		0	1	100										
16		0		2	2	—		1		1	2	50		2		1	3	67										
18		0		3	3	—		0		0	0	—		1		2	3	33										
20		0		1	1	—		0		0	0	—		1		0	1	100										
22		1		2	3	33		0		0	0	—		0		0	0	—										
24		0		1	1	—		0		0	0	—		0		0	0	—										
26		1		0	1	100		0		0	0	—		1		0	1	100										
Total B Av.		2		25	27	7		3		9	12	25		8		11	19	42										
Total A plus B Av.		59		26	85	69		71		12	83	86		60		22	82	73										
Neomycin, (30-30)																			Polymyxin, (50-25)					Tetracycline, (5-5)				
A 6	15		3		18	83	8		5		13	62	81		1		82	99										
8	5		0		5	100	3		8		7	27	0		0		0	—										
10	3		8		11	27	6		27		33	18	0		1		1	—										
Total A Av.	23		11		34	68	17		40		57	30	81		2		83	98										
B 12	16		5	21	76	—	16		4	20	80	80	0		1	1	1	—										
14	9		2	11	82	—	4		1	5	80	80	0		1	1	1	—										
16	10		0	10	100	—	0		0	0	—	—	0		0	0	0	—										
18	4		0	4	100	—	0		0	0	—	—	0		0	0	0	—										
20	3		0	3	100	—	0		0	0	—	—	1		0	1	100											
22	1		0	1	100	—	0		0	0	—	—	0		0	0	0	—										
24	0		0	0	—	—	0		0	0	—	—	0		0	0	0	—										
26	0		0	0	—	—	0		0	0	—	—	0		0	0	0	—										
Total B Av.	43		7	50	86	86	20		5	25	80	80	1		2	3	33											
Total A plus B Av.	66		18	84	79	79	37		45	82	45	45	82		4	86	95											

* RR = resistant by both methods; RS = resistant by the disc method, susceptible by the pour plate dilution method; SS = sensitive by both methods; SR = sensitive by the disc method, resistant by the pour plate dilution method.

† The first number in parentheses expresses antibiotic content of disc. The second number expresses the concentration of antibiotics in $\mu\text{g./ml.}$ of medium.

situation would hold for the two penicillin concentrations and staphylococci. It has been mentioned previously, in the case of polymyxin B and *Pseudomonas* species, that a choice of a minimum zone size of 10 rather than 12 mm. increases the agreement between the two methods from 46 per cent for the latter zone size to 70 per cent for the former zone size. However, if one continues to analyze the data from this viewpoint, it becomes evident that the agreement between the methods is: (1) not significantly altered with a different choice of minimum zone size; or (2) is altered with the result that the agreement decreases appreciatively. The latter situation would rule for the majority of the results presented in this study (see vancomycin and novobiocin, table I). On the other hand, with the possible ex-

ception of the staphylococci, there may be a question concerning the significance of the number of organisms tested for each genera or species and, therefore, whether a significant statement may be made regarding the choice of a minimum zone size as a criterion of sensitivity to an antibiotic.

In conclusion, the agreement of the results, derived from a comparative study of the use of the paper disc and plate dilution methods for determining the in vitro susceptibility of 710 organisms representing six genera, is good.

SUMMARY

A comparative study of the use of the paper disc and plate dilution methods for the routine testing of the susceptibility to various antibiotics of 710 bacterial cultures representing six genera has been made. With few exceptions the agreement of results between the two methods is good. Selection of a minimum zone size for each antibiotic as a criterion for sensitivity of the organisms to the individual antibiotics has been considered and discussed.

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Comparison of the In Vitro Activity of Four Polyenic Antifungal Antibiotics

HUBERT LECHEVALIER

Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J.

Numerous actinomycetes produce polyenic antifungal antibiotics.^{4,5} These antibiotics are characterized by typical light absorption spectra, which are caused by conjugated carbon-to-carbon double bonds.^{3,6} Three of these polyenes have found a place in chemotherapy: trichomycin, nystatin, and amphotericin B.

The present study provides a comparison of the in vitro antifungal activity of one tetraene, nystatin, and three heptaenes, candicidin, candidin, and amphotericin B.

The data show that best activity was obtained against growing fungi at a neutral or slightly alkaline reaction. In contrast, the killing of yeast cells in a phosphate buffer was more marked when it was acidic.

MATERIALS AND METHODS

Stock solutions of pure nystatin (lot HV 617), a lot of amphotericin B (pure crystalline), pure candidin (lot 1560), and 80 per cent pure candicidin (lot 880) were made in dimethylsulfoxide at the level of 1 mg./ml. Further dilutions were made in water. Dilutions were made to get the following final concentrations: 1, 2, 3, 5, 7, and 10 µg./ml., or similar values in a different logarithm range, such as: 0.01, 0.02, 0.03, 0.05, 0.07, and 0.1 µg./ml. The concentrations used depended on the sensitivity of the test organisms under the conditions of the assay. Assays were made in peptone-meat extract-glucose medium (1 per cent glucose, 0.5 per cent peptone, 0.5 per cent sodium chloride, 0.3 per cent meat extract, pH 6.6 after sterilization). This medium was buffered when necessary with appropriate mixtures of *M*/20 potassium phosphate salts and was used either as a solid or as a liquid. The list of the test organisms used will be found in table I. Stocks of the organisms were grown on yeast extract-glucose agar and were incubated 18 hours at 28 C. for the yeast and 48 hours at the same temperature for the filamentous fungi. The same incubation procedure was used in the assays. Turbidimetric measurements were made with a Klett-Summerson colorimeter using a green filter (540 mµ). Determination of fungicidal activity was accomplished by placing the antibiotics in contact with twice-washed cells of *Saccharomyces cerevisiae* in *M*/20 potassium phosphate buffer. After the period of contact, the cells were again washed twice with water and plated on peptone-meat extract-glucose agar for viability counts.

RESULTS AND DISCUSSION

Only a few test organisms are required to demonstrate differences in the antifungal spectra of these four antibiotics. Preliminary surveys were made with many

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TABLE I

Effect of 4 Polyenes Against Four Fungi, Tested by Streak Dilution Assay on Peptone-Meat Extract-Glucose Agar

Antibiotic	Minimum inhibitory concentration, $\mu\text{g./ml.}^*$			
	<i>Candida albicans</i> 204	<i>Saccharomyces cerevisiae</i> 216	<i>Aspergillus niger</i> 13	<i>Mucor rouxii</i> 80
Nystatin	1.2	0.8	2.0	0.8
Candidin	0.17	0.17	0.4	0.2
Amphotericin B	0.2	0.2	0.12	0.06
Candididin	0.02	0.01	0.3	0.3

* Average of three assays.

different fungi. The four organisms used in the data reported in table I were selected for the practical differentiation of these four substances. As can be seen in the table, amphotericin B was the most active compound used against the filamentous fungi; nystatin was the least active. Against yeasts, there was more variation; candididin was about 10 times more active than either candidin or amphotericin B. These antibiotics in turn were about five times more active than nystatin.

During the preliminary survey of the antifungal spectra of candidin and amphotericin B, it was found difficult to differentiate between these two antibiotics. They seemed to have identical biological properties and are chemically quite similar. A few strains of *Aspergillus niger* and a strain of *Mucor rouxii* made the distinction between these two antibiotics possible. As can be seen in table I, candidin and amphotericin B had the same activity against *Candida albicans* and *S. cerevisiae*, but amphotericin B was about three times more active against *A. niger* and *M. rouxii*.

Lampen and co-workers¹ have shown that nystatin inhibited the endogenous respiration and the aerobic and anaerobic utilization of glucose by *S. cerevisiae*, *Penicillium chrysogenum*, and other fungi. This inhibition was more effective at an acid pH (4.5) than at neutrality. From this, one should not conclude that polyenes in general and nystatin in particular are most antifungal under all circumstances

TABLE II

Effect of 4 Polyenic Antifungal Antibiotics on Solid Peptone-Meat Extract-Glucose Medium at 2 Different pH Values

Antibiotic	Minimum inhibitory concentration, $\mu\text{g./ml.}^*$						For <i>C. albicans</i> Ratio pH 4.5/ pH 7
	<i>C. albicans</i>		<i>S. cerevisiae</i>		<i>A. niger</i>		
	pH 4.5	pH 7	pH 4.5	pH 7	pH 4.5	pH 7	
Nystatin	4.5	1.6			5.7	1.7	2.8
Candidin	0.5	0.12	0.40	0.11			4.2
Amphotericin B	0.5	0.15	0.43	0.09			3.3
Candididin	0.057	0.005	0.025	0.001	0.7	0.1	11.5

* Average of three assays.

TABLE III

Effect of 4 Polyenes Against Saccharomyces cerevisiae in Liquid Peptone-Meat Extract-Glucose Medium at 2 Different pH Values

Antibiotic	$\mu\text{g./ml.}$ necessary to reduce growth to 50 per cent of controls*		Ratio $\text{pH } 4.5/\text{pH } 7$
	pH of medium		
	4.5	7†	
Nystatin	0.32	0.39	0.8
Candidin	0.070	0.034	2.1
Amphotericin B	0.068	0.031	2.2
Candididin	0.0051	0.0011	4.6

* Average of three assays.

† pH did not fall below 6.8.

at an acid pH. Many other phenomena should be considered. For instance, the polyenes studied were more soluble at a neutral or an alkaline pH than at pH 4.5. Moreover, in general, fungi grow better at an acid pH than at neutrality and could therefore possibly be less susceptible to the action of drugs at pH 4.5 than at pH 7.0.

The net results of the action of these various factors are brought out in tables II and III. On solid peptone-meat extract-glucose agar, buffered at pH 4.5 and 7.0, all the organisms tested were by far more sensitive to the action of polyenes at pH 7 than at pH 4.5. This pH effect was most striking with candididin. In the same medium used in shallow layers (10 ml. of medium in 250 ml. Erlenmeyer flasks), the pH effect was much less marked; in the case of nystatin, hardly any difference was noted between both pH values. Differences in pH response between the solid and liquid media under rather similar conditions of aeration suggested that diffusion phenomena may play a role on the solid medium. As the antibiotics were used up in the seeded area of the plates, more antibiotics had to diffuse from the surrounding medium to check the growth of survivors. This was easier when the antibiotics were in a more soluble state, namely, at a neutral or alkaline pH.

A completely different picture was observed when the same antibiotics were put

TABLE IV

Fungicidal Action of 4 Polyenes Against Cells of Saccharomyces cerevisiae Suspended in Phosphate Buffers

Antibiotic	Conc., $\mu\text{g./ml.}$	Percentage of survivors*	
		pH	
		4.4 to 4.6	7.4 to 7.8
Nystatin	10	2	55
Candidin	10	1	5
Amphotericin B	10	0.4	10
Candididin	1	0.03	0.07

* After four to six hours' exposure to the antibiotics at 28 C. Average of three experiments.

in contact for four to six hours with washed cells of *S. cerevisiae* in potassium phosphate buffers. As can be seen in table IV, cells died in greater number when exposed to the action of 10 µg./ml. of nystatin, candidin, or amphotericin B at pH 4.4 than at pH 7.8. Candidin, at the concentration used (1 µg./ml.), was the most potent fungicidal agent of the polyenes tested. In this case, the effect of pH was not striking.

Nystatin was a remarkable fungicidal agent. At pH 4.5, its action was of the same magnitude as that of candidin and amphotericin B. However, it had less fungicidal action at pH 7.8. These data are similar to those reported by Lampen and co-workers.²

Differences in the pH optima for the inhibition of growing cells and for the killing of resting cells may be due, as already mentioned, to the greater vigor of the growing fungi at an acid pH. This might shift the optimum pH of the drugs from an acid pH toward neutrality. The greater solubility of the polyenes at an alkaline pH may also be a factor. However, one should not discard the hypothesis that polyenes may have multiple sites of action. One such site, for example, the inhibition of the formation of a cellular constituent, could be of extreme importance in preventing growth. This hypothetical site of action might be more easily inhibited at a neutral or alkaline reaction than at an acid pH. On the other hand, the killing of nongrowing cells could be caused by the action of the drugs on a different site, most effectively disrupted at an acid reaction.

SUMMARY

1. The main facets of the antifungal spectra of nystatin, candidin, amphotericin B, and candidin are reviewed. In vitro, nystatin was the least active compound against growing fungi, in general, whereas candidin was the most active compound against yeasts.

2. Candidin and amphotericin B were found to have almost identical antifungal spectra. The two closely related antibiotics were differentiated biologically with the help of four test organisms.

3. The four polyenes, when tested on a solid medium, were more active against growing fungi at a neutral pH than at pH 4.5. In a liquid medium, differences in activity at various pH values were less striking. In all cases the activity of candidin varied with pH more than that of the other three polyenes. In contrast, the activity of nystatin was the least affected by pH variations.

4. The fungicidal action of nystatin, candidin, and amphotericin B on cells of *Saccharomyces cerevisiae* in phosphate buffers was more pronounced at pH 4.4 than at pH 7.8. Candidin was the most potent fungicidal agent tested, and, under the conditions used, its action was about the same at both pH values.

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crystalline amphotericin B. Dr. Edward Borowski kindly supplied samples of pure candidin (lot 1560) and 80 per cent pure candicidin (lot 880).

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The Effect of Amphotericin on the Respiration of *Cryptococcus*

EVERETT R. RHOADES* AND HAROLD G. MUCHMORE†

*Department of Medicine, Oklahoma University School of Medicine and
Veterans Administration Hospital, Oklahoma City, Okla.*

Before the discovery of amphotericin B‡ and the demonstration of its usefulness in the treatment of certain of the deep mycoses, no effective agent was available for the treatment of cryptococcosis. Clinical studies¹⁻⁴ have shown that amphotericin B is useful in the treatment of cryptococcosis as well as other fungus infections. Before the advent of amphotericin B, few studies of the metabolic processes of *Cryptococcus neoformans* were reported.⁵ Other than the clinical improvement and the inhibition of growth, nothing is reported regarding the mechanism of action of this substance on *Cryptococcus*. In this study, aerobic metabolism of *C. neoformans* and the effect of amphotericin B on this process were investigated.

METHODS

Three strains of *C. neoformans* obtained from patients at the University of Oklahoma Medical Center were studied by conventional Warburg technique.⁶ Each strain of the organism was grown at room temperature in a commercial liquid medium§ and subcultured every two weeks. For the Warburg studies the organisms

§ The trade name of Difco Laboratories for this medium is Difco Mycological Broth.

were grown in low form culture flasks containing 300 to 400 ml. of the same medium and harvested and washed by repeated centrifugation. The cells were washed in *M*/15 phosphate buffer, pH 7.0, until free of glucose, measured by the glucose oxidase method.⁷ One ml. of this cell suspension of 5 to 15 mg./ml. dry weight was used in each Warburg flask. The oxygen uptake was measured at 37 C. in the Warburg apparatus utilizing 0.278 *M* glucose as a substrate alone and in the presence of varying concentrations of amphotericin B. The antibiotic used was the standard commercial preparation, which contains sodium desoxycholate as a vehicle. The final concentrations of amphotericin B used were 1.0, 3.0, and 10.0 µg./ml. Appropriate concentrations of sodium desoxycholate were tested alone for its action because of its presence in the preparation used. The QO_2 values were determined in the usual manner and represented as microliters of oxygen taken up by 1 mg. dry weight of organisms in one hour.

RESULTS

A typical experiment is represented graphically in figure 1. The results of the manometric studies are summarized in table I. With 0.278 *M* glucose as the sub-

* Captain, USAF (M.C.). The contents of this article are the personal views of the Air Force author and are not to be construed as statement of official Air Force policy.

† Clinical Investigator, Veterans Administration Hospital, Oklahoma City, Oklahoma.

‡ The trade name of E. R. Squibb and Sons Division, Olin Mathieson Chemical Corp., for amphotericin B is Fungizone.

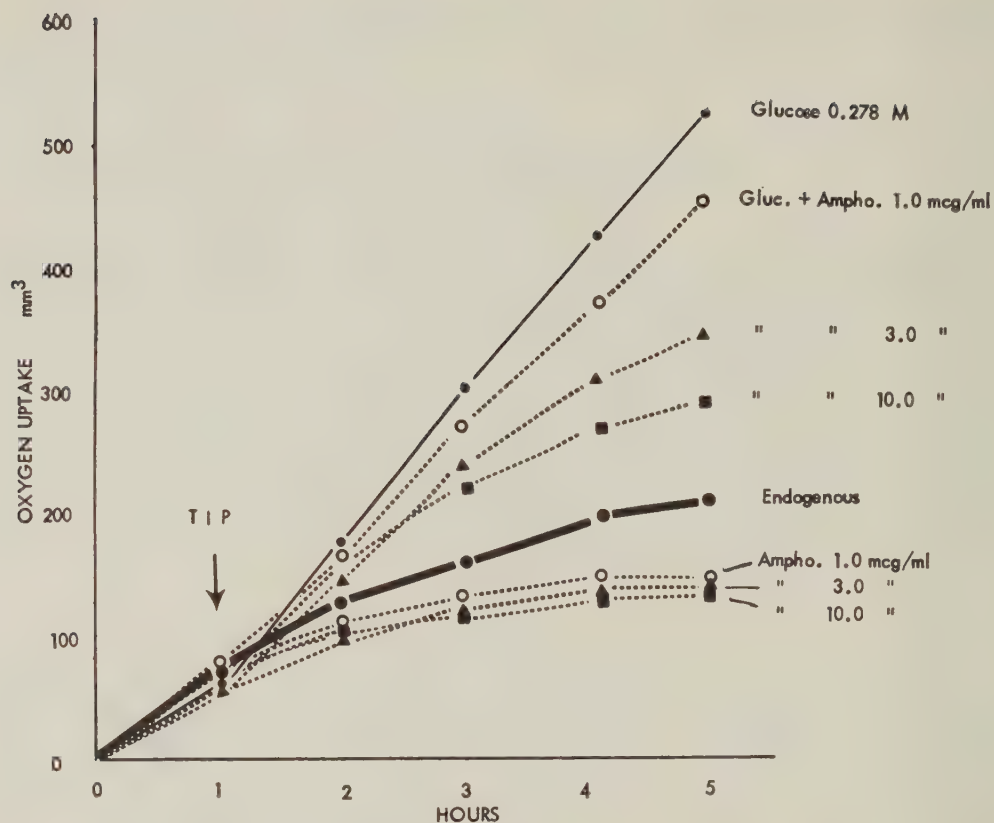


FIG. 1. Oxygen uptake at 37 C. of resting *C. neoformans* cells suspended in *M/15* phosphate buffer in presence of amphotericin B alone, glucose alone, and amphotericin B and glucose together. The dry weight of the cells is 13.7 mg./ml. The substrate contained in the flask side arm was mixed with the cell suspension after one hour as indicated by the arrow marked "tip."

strate, the oxygen uptake was increased an average of 55 per cent over the endogenous rate. This increase was significant at the 5 per cent confidence level. The addition of 1.0, 3.0, and 10.0 $\mu\text{g./ml.}$ final concentration of amphotericin B to the cell suspension caused a decrease in the oxygen uptake below the endogenous rate. This decrease was proportional to the concentration of amphotericin B used and was statistically significant within the 5 per cent confidence limit in a concentration as low as 1.0 $\mu\text{g./ml.}$ When the same final concentrations of amphotericin B were tested with 0.278 *M* glucose, the oxygen uptake of the cell suspension was decreased below that observed with glucose alone. This decrease was proportional to the amphotericin B concentration and was significant at the 5 per cent level. Similar results were obtained with all three strains of *C. neoformans* tested. No effect on the oxygen uptake was noted with sodium desoxycholate.

DISCUSSION

These preliminary data show that amphotericin B, in the concentrations likely to be achieved in clinical use, causes a significant decrease in the oxygen uptake of

TABLE I

Change of QO_2 with Glucose and Various Concentrations of Amphotericin B

Substrate	No. of experiments	Endogenous, mean QO_2	Experimental, mean QO_2	Change, mean QO_2	% change
Glucose, 0.278 M	17	9.2	13.2	+ 4.9	↑ 53
Glucose, 0.278 M, plus amphotericin, 1.0 $\mu\text{g.}/\text{ml.}$	10	7.7	10.1	+ 2.5	↑ 32
Glucose, 0.278 M, plus amphotericin, 3.0 $\mu\text{g.}/\text{ml.}$	4	5.3	7.2	+ 1.9	↑ 36
Glucose, 0.278 M, plus amphotericin, 10.0 $\mu\text{g.}/\text{ml.}$	10	8.6	7.9	- 0.8	↓ 9
Amphotericin, 1.0 $\mu\text{g.}/\text{ml.}$	10	7.3	4.9	- 2.4	↓ 24
Amphotericin, 3.0 $\mu\text{g.}/\text{ml.}$	4	5.3	4.0	- 1.3	↓ 32
Amphotericin, 10.0 $\mu\text{g.}/\text{ml.}$	9	7.3	4.2	- 3.1	↓ 42

resting *C. neoformans* cells. Seabury and Dascomb² reported that 0.06 to 0.6 $\mu\text{g.}/\text{ml.}$ amphotericin B will inhibit in vitro growth of *Cryptococcus*. They further indicated that intravenous administration of amphotericin B will result in a blood concentration of 1 $\mu\text{g.}/\text{ml.}$ for 24 hours after an infusion.² It is reasonable that the concentration of 1 $\mu\text{g.}/\text{ml.}$ used in this study approximates that used clinically. Because of the decreased uptake of oxygen, it is possible that part of the activity of amphotericin B against *Cryptococcus* is due to interference with aerobic glycolysis.

SUMMARY

Amphotericin B in concentrations of 1.0 to 10.0 $\mu\text{g.}/\text{ml.}$ will decrease the oxygen uptake of resting *C. neoformans* cells below endogenous levels. The increase of oxygen uptake produced by the addition of glucose to the resting cells is hindered by the presence of amphotericin B in the same concentrations.

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Beneficial Therapeutic Effects of Solubilized Amphotericin B after Oral Administration in Experimental Coccidioidomycosis, Histoplasmosis, and Cryptococcosis in Mice

CHARLOTTE C. CAMPBELL* AND GRACE B. HILL†

*Walter Reed Army Institute of Research, Walter Reed Army Medical Center,
Washington, D. C.*

At the present time amphotericin B‡ is perhaps the most promising drug available for the treatment of mycotic infections in human beings. Unfortunately, and in spite of a few reports to the contrary, it is considered ineffective after oral administration.¹⁻⁷ Intramuscular injection not only is said to be ineffective but is attended by painful indurated tumescences at the site of injection.⁴ The drug thus is usually given by infusion, a time-consuming process involving the slow intravenous drip of 500 ml. glucose solution containing only 10 to 50 mg. amphotericin B over a period of six hours.³⁻⁵ This process, moreover, is repeated every other day, or three times weekly, for four to six months. In many patients, infusion is accompanied by such distressing reactions as fever, shaking chills, nausea, vomiting, vestibular disturbances, headache, and elevated blood urea nitrogen, all fortunately transitory.³⁻⁵ Nevertheless, because of the severity of these reactions, some patients refuse to take the drug, and a less painful and time-consuming procedure for administering it would be rewarding for both patient and clinician.

Paradoxically, the oral route has been shown repeatedly to be effective in controlling mycotic infections in mice, although total dosages of 375 mg./Kg. were required to prolong survival even when therapy was initiated as early as one day after infection. Higher dosages were needed to eradicate the infective agents.^{4,8,9} These earlier experiments were carried out with water-insoluble preparations of amphotericin B, or the same type of preparation that has been administered orally to patients, usually without success.

In contrast the amphotericin B employed for infusion into human beings is a special preparation, which is previously solubilized with sodium desoxycholate. After the addition of water, it becomes a colloidal suspension with fine, evenly dispersed particles.¹⁰ Reasoning that this very physical property might make the drug more readily absorbable from the gastrointestinal tract, a series of experiments in mice infected with experimental histoplasmosis, coccidioidomycosis, and cryptococcosis and treated orally with the solubilized type of preparation were undertaken. The concentrations required to prolong survival time and to eradicate these infective agents from tissues were determined and compared with the concentrations of nonsoluble preparations needed to achieve equally good results. The role

* Department of Bacteriology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

† Veterans Administration Central Laboratory for Clinical Pathology and Research, and Department of Bacteriology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

‡ The trade name of E. R. Squibb & Sons Division, Olin Mathieson Chemical Corp., for amphotericin B is Fungizone.

of assayable serum levels and the possibility of obtaining improved results in man by the oral administration of this type of preparation are discussed.

MATERIALS AND METHODS

Strain G-8 of *Histoplasma capsulatum*, D-1 of *Cryptococcus neoformans*, and C-23 of *Coccidioides immitis*, all isolates from cases in human beings, were employed. The inoculating suspensions for *H. capsulatum* were prepared from 48 hour yeast phase cultures incubated at 37 C. on brain-heart infusion agar. The organisms were harvested, washed once, resuspended in cold physiological saline solution, and centrifuged at 350 r.p.m. for three minutes to remove aggregates of cells.¹¹ The supernatant suspension was further diluted to transmit 40 per cent light at 500 γ (Coleman spectrophotometer, model 14). Suspensions of *C. neoformans* were prepared similarly from 48 hour cultures incubated at room temperature on Sabouraud's agar. After adjustment to 50 per cent light transmission at 500 r. in physiological saline solution, these suspensions were further diluted 1:4 for injection into mice. *C. immitis* inoculating suspensions were prepared from 10 day cultures of the filamentous growth phase of the organisms on Sabouraud's agar and, after mechanical disruption of the filaments, diluted to contain 130 to 150 viable particles/ml. physiological saline solution. For each suspension, the inoculum per mouse was 0.2 ml., which was administered by the intravenous route. In addition, inoculating suspensions of the two yeast species were maintained in an ice bath throughout the period of inoculation.¹¹

The amphotericin B employed was the colloidal preparation designed for infusion into human beings. After initial "solution" in sterile distilled water to a concentration of 1 mg./ml., the drug was added to measured volumes of the animals' drinking water in concentrations varying from 0.0025 to 0.05 mg./ml. and was consumed ad libitum. Fresh drug was supplied at 48 hour intervals during the course of therapy, and the quantities consumed were carefully tabulated. The average total quantity consumed per mouse was calculated at the end of the course of therapy.

Male albino mice weighing 16 to 18 Gm. were used in groups of 10 and 20. Treatment was begun at the time of infection or at 2, 4, 8, or 10 days thereafter. Controls used for each experiment were groups of mice infected and provided only with water, uninfected and provided only with water, and uninfected and provided with water containing the concentrations of drug used in the experiment. Animals were observed daily from 28 to 60 days and the number of deaths recorded. All untreated infected mice were autopsied on the day of death and their spleens cultured for the infective agents. Similar cultural studies were made on the spleens of treated survivors selected at random for sacrifice at various intervals after time of infection as noted in the separate experiments.

Evaluation of therapeutic activity was based on the prolongation of survival time of treated as compared with untreated animals and on the isolation of the infective organism from autopsied mice of treated and untreated groups.

To determine whether the solubilized amphotericin B preparation was more readily absorbed from the gastrointestinal tract than nonsolubilized preparations, healthy mice were allowed to consume ad libitum daily dosages of 2.5, 25, 250, and 500 mg./Kg. The sera from 4 mice in each group were collected and pooled at

TABLE I

*Therapeutic Effect of Solubilized Amphotericin B after Oral Administration
in Mice Experimentally Infected with H. capsulatum,
C. neoformans, and C. immitis*

Organism	Total drug, mg./Kg.	Per cent survival on sixtieth day	Cultures on day		
			14	28	42
<i>H. capsulatum</i>	40, 80, 160	100	—	—	—
<i>C. neoformans</i>	40, 81, 162	100	—	—	—
<i>C. immitis</i>	42, 85, 170	100	—	—	—
Untreated infected controls	0, 0, 0	0-5	+	+	
Treated uninfected controls	40, 80, 170	100			

24 hour intervals for four days and assayed for amphotericin B according to the method described by Taylor et al.¹² A strain of *Saccharomyces cerevisiae* with a minimal inhibitory concentration of 0.075 mg./ml. was used as the test organism, and the solubilized preparations of amphotericin B employed in the experiments as the standard for determination of the minimal inhibitory concentration.

RESULTS

As shown in table I, the oral administration of solubilized amphotericin B provided a percentage survival of 100 in mice experimentally infected with *C. immitis*, *H. capsulatum*, and *C. neoformans* throughout a 60 day observation period. In contrast, all untreated infected mice were dead by the twenty-eighth day. There were 20 mice in each of these groups. Therapy was started within three hours after infection and continued for 10 days to yield total dosages of approximately 40, 80, and 160 mg./Kg. for each infective agent. It will be noted, moreover, that cultures



FIG. 1. The effect of solubilized amphotericin B in mice infected with *Cryptococcus neoformans* following a daily dosage of 7 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

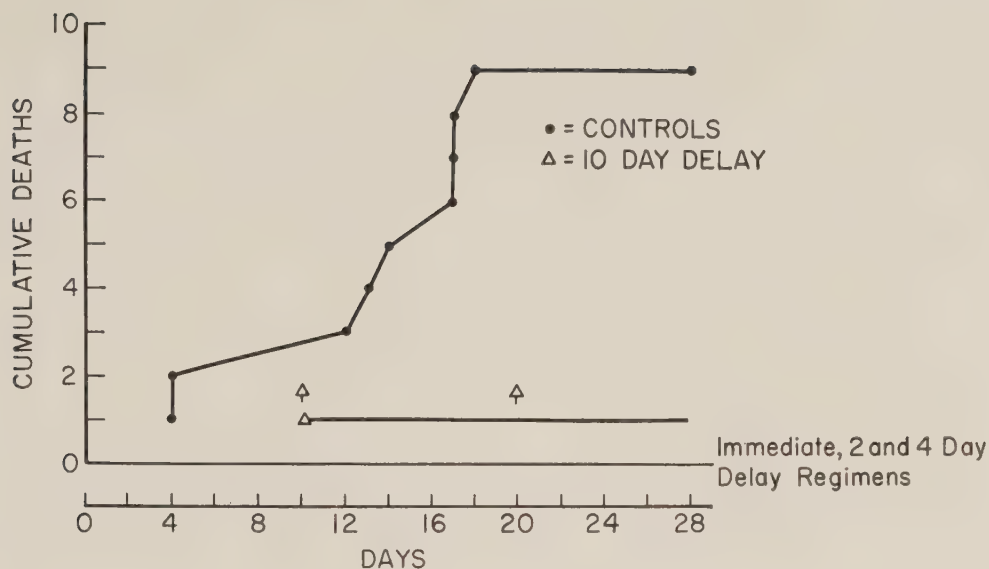


FIG. 2. Effect of solubilized amphotericin B in mice infected with *Coccidioides immitis* following a daily dosage of 7 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

of spleens from selected treated mice sacrificed 14, 28, and 42 days after infection were negative for these agents, whereas those from all untreated infected mice were positive at autopsy. Finally, even the highest dosage not only was readily consumed but was well tolerated by the mice. There were no deaths in any of the drug controls throughout the entire series of experiments described in this report.

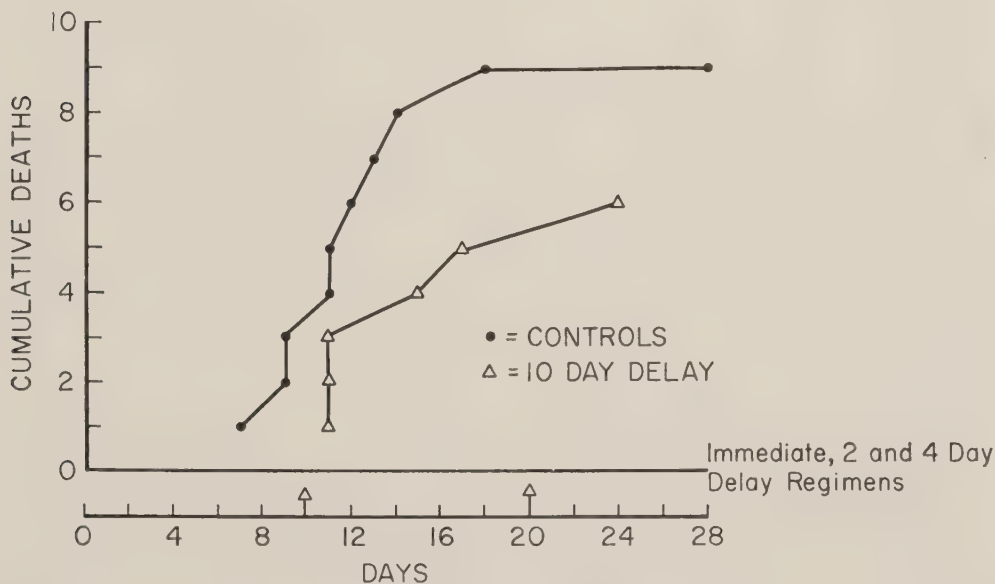


FIG. 3. Effect of solubilized amphotericin B in mice infected with *Histoplasma capsulatum* following a daily dosage of 7 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

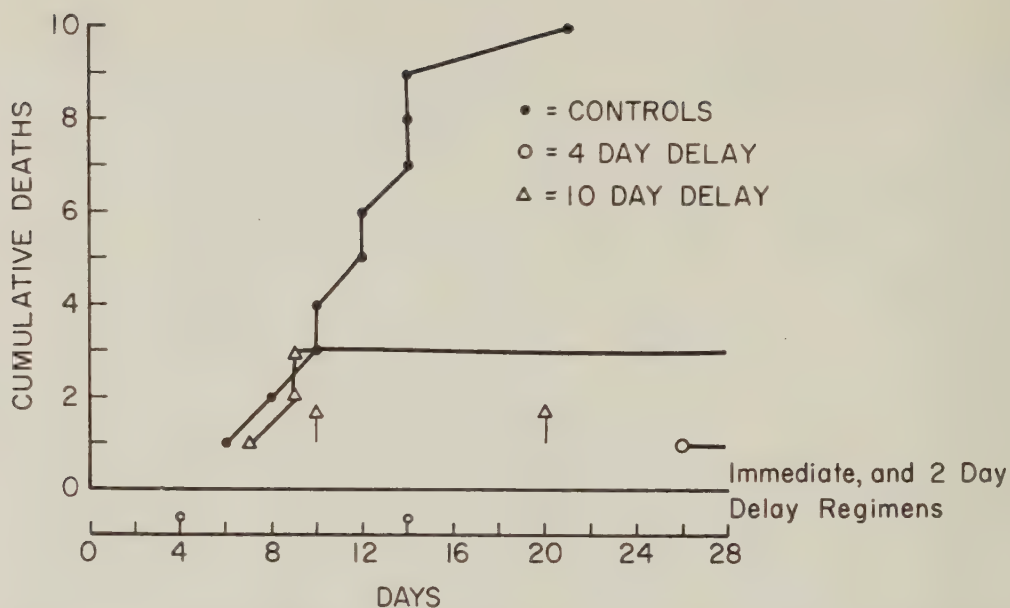


FIG. 4. The effect of solubilized amphotericin B in mice infected with *Cryptococcus neoformans* following a daily dosage of 1.75 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

The complete protection afforded by these small total dosages when therapy was started at time of infection led to additional experiments with lower concentrations of solubilized drug. In these studies, 10 mice were used in each group and therapy was initiated not only at the time of infection but at 2, 4, 8, and 10 day intervals

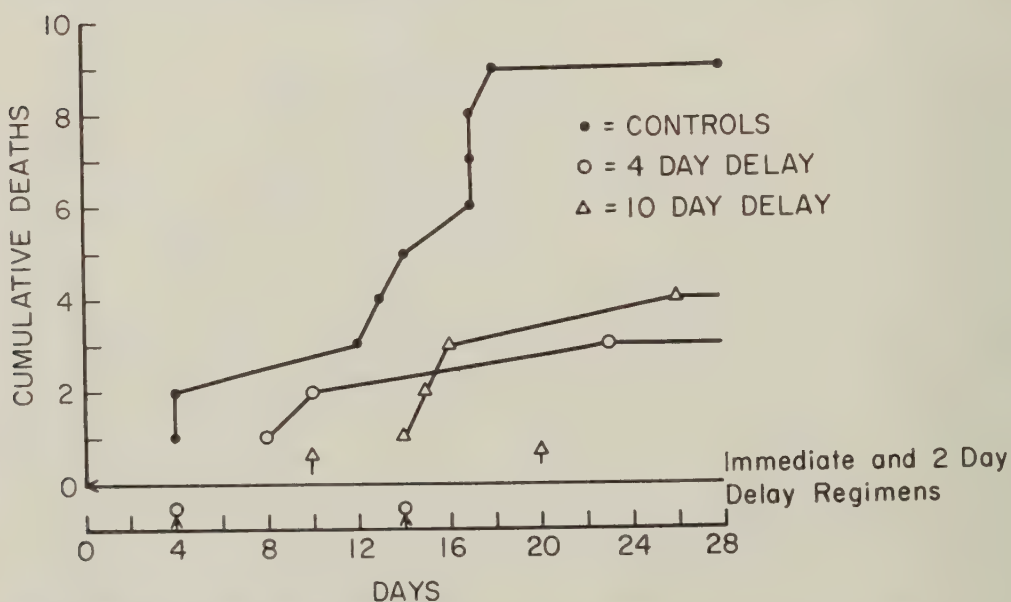


Fig. 5. Effect of solubilized amphotericin B in mice infected with *Coccidioides immitis* following a daily dosage of 1.75 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

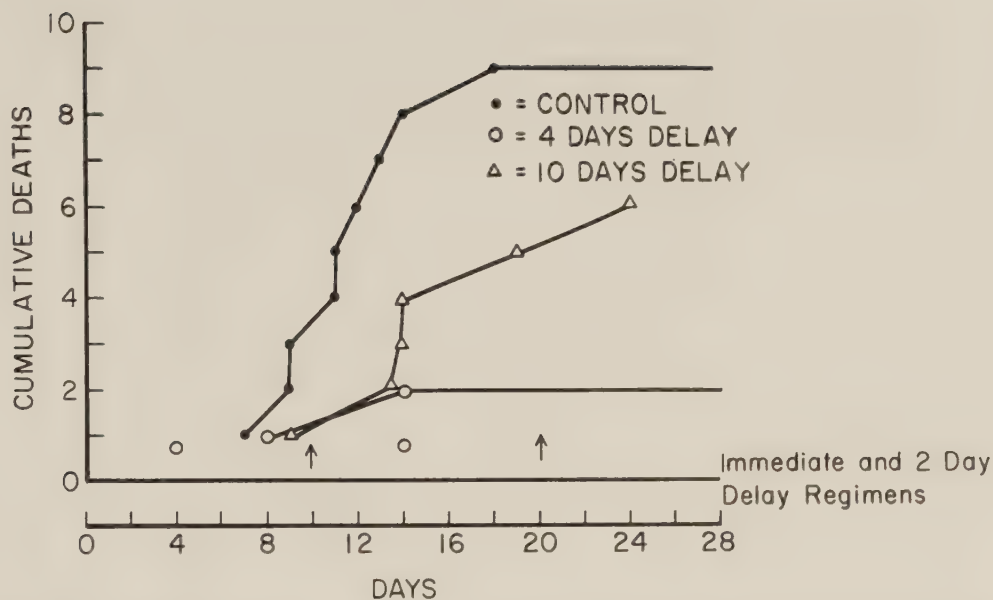


FIG. 6. Effect of solubilized amphotericin B in mice infected with *Histoplasma capsulatum* following a daily dosage of 1.75 mg./Kg./day for 10 consecutive days in immediate and delayed therapy regimens.

thereafter and continued for 10 days. Animals in this series were observed for a total period of 28 days. Total dosages ranged from 4 to 7 to 60 to 70 mg./Kg.

As shown in figure 1, there were no deaths in mice with experimental cryptococcosis treated with 7 mg./Kg./day even when therapy was withheld for as long as 10 days. The advanced state of the infection at that time is indicated by the 30 per cent fatality in infected untreated controls. Results in experimental coccidioidomycosis, shown in figure 2, were similar except that in this series only 15 per cent of the infected untreated mice were dead when treatment with 7 mg./Kg./day was begun on the tenth day. In experimental histoplasmosis the infection was far advanced when therapy was initiated on the tenth day. As illustrated in figure 3, three of the six fatalities in mice on this regimen occurred after only one day on the drug. However, there was 100 per cent survival in mice treated as late as the fourth day.

The results of simultaneous studies carried out with approximately one fourth these daily dosages of solubilized amphotericin B, or 2 to 2.5 mg./Kg., are shown in figures 4, 5, and 6. In this instance, there was 100 per cent survival only in those mice treated immediately and two days after infection. As shown in figure 4, there was one death in treated animals with experimental cryptococcosis, and this occurred in mice first treated on the fourth day of disease. In experimental coccidioidomycosis, shown in figure 5, there were two deaths in treated mice receiving the full course of drug, one each in the 4 and 10 day delayed regimens. In experimental histoplasmosis, 2 mice died during the course of the four day delayed and 4 during the 10 day delayed regimens (fig. 6). This minimal dosage, therefore, clearly was insufficient to control all actively advancing infections of experimental histoplasmosis, coccidioidomycosis, or cryptococcosis. Nevertheless, in all instances, the time of survival of mice that lived to receive the full course of drug was

markedly prolonged over that of untreated mice, a high percentage of which were dead by the twenty-first day.

The 4 to 7 mg./Kg. total dose failed to prolong the survival of all mice treated at the time of infection. Conversely, results obtained with a total dosage of 35 mg./Kg. were essentially as good as those with 70 mg./Kg. For these reasons, only the data obtained with total dosages of 17.5 and 70 mg./Kg. were illustrated. The data for the eight day delay regimens were also omitted from the illustrations, since they were remarkably similar to those obtained in the 10 day delay schedules.

For the series of experiments depicted in figures 1 to 6, cultural studies were carried out only on the twenty-eighth day. One mouse randomly selected from the survivors of each treated group was sacrificed, and its spleen cultured for the respective infective agent. Only one positive culture was observed, and this was found in an animal infected with *C. immitis* first treated on the tenth day after infection with the minimal 17.5 mg./Kg. total dosage of drug. In contrast, the spleens of all untreated animals were culturally positive when autopsied on the day of death.

The sera from normal healthy mice treated orally with 2.5, 25, and 250 mg./Kg./day solubilized amphotericin B in drinking water were assayed after 24, 48, and 96 hours of drug. Drug levels were not demonstrated in any of these sera by the forty-eighth hour after start of therapy. By the ninety-sixth hour, however, the sera from mice receiving total dosages of 100 and 1000 mg./Kg. each contained 1.20 µg./ml. amphotericin B, while those receiving a total dosage of only 10 mg./Kg. contained 0.32 µg./ml.

DISCUSSION

The results of studies described herein indicate that the solubilized preparation of amphotericin B is highly effective in controlling mycotic infections in mice after administration by the oral route. In experimental cryptococcosis, histoplasmosis, and coccidioidomycosis, a total dosage of as little as 70 mg./Kg. was sufficient not only for prolonging the survival of all animals first treated as late as the fourth and in some instances the tenth day after infection, but also for producing negative cultures in these animals. That the solubilized preparation is also readily absorbed from the gastrointestinal tract of mice is shown by the serum levels of 0.32 µg./ml. demonstrated as early as the fourth day after daily oral doses of 2.5 mg./Kg., or a total dosage of 10 mg./Kg. After four daily doses of 25 and 250 mg./Kg., or total dosages of 100 and 1000 mg./Kg., respectively, the serum levels were increased to 1.2 µg./ml.

These results were superior to those obtained earlier in this laboratory with a nonsolubilized preparation of drug (lot no. SRAM-27X-2Cr).⁹ In a typical experiment, a total dosage of 175 mg./Kg. given orally over a 10 day period beginning at the time of infection failed even to prolong the survival time of mice infected with *C. neoformans*. Nor were demonstrable serum levels developed at any time during or within 48 hours after this course of therapy. Louria et al,⁸ employing another lot of a nonsolubilized preparation (lot no. SDAM-638-Gx), found that a total dosage of 375 mg./Kg. started one day after infection with *C. neoformans* and continued for 25 consecutive days prolonged the survival time of the mice but failed to produce culture negativity. Again, serum levels were not demonstrable.

These findings are in contrast to those reported herein in which infections were controlled, if not completely eradicated, with total dosages of as little as 17.5 mg./Kg. of the solubilized preparation of amphotericin B, even when therapy was initiated as late as the tenth day after infection (fig. 4).

These results suggest that the oral administration of solubilized preparations of amphotericin B might also be more effective in controlling mycotic infections in man than the nonsolubilized preparations currently used for this purpose. Failure to demonstrate assayable amounts of amphotericin B in the patient's serum after oral therapy is the reason usually advanced for therapeutic failures in persons receiving the nonsolubilized preparations by this route.³⁻⁵ It is reasonable to assume that the solubilized preparations might also be more readily absorbed by man, as they are by mice.

There is considerable evidence, moreover, to indicate that very low levels of drug are required to control many of the mycotic infections in man. Serum levels were never demonstrated during successful oral therapy of a case of musculoskeletal disseminated coccidioidomycosis of 10 years' duration reported by Klapper et al.¹ nor in a second disseminated case of this infection described by Fiese.² While it is possible that both of these cases were caused by strains of *C. immitis* that were exquisitely sensitive to the drug, it is also to be noted that direct infusion of 50 to 75 mg. of the solubilized preparation is rarely accompanied by levels exceeding 1.5 to 2.0 µg./ml. during the period of infusion and that these are completely diminished by 48 hours after discontinuance thereof.³ The studies reported herein reveal that levels approaching this magnitude were demonstrated in mice after oral administration of four 25 mg./Kg. daily doses of the solubilized preparation. Should this type of preparation prove to be as rapidly absorbed by the gastrointestinal tract of man, similar levels could be not only achieved but continuously maintained in the average person with a daily dosage of as little as 1.75 Gm. a day.

Finally, it is worthy of note that some of the patients failing to respond to oral therapy with the nonsolubilized preparations were moribund when treatment was started. Such cases appear in the series reported by Newcomer et al.,⁴ Littman et al.,³ and Fitzpatrick et al.⁵ The purpose of this report therefore is not to suggest the discontinuance of infusion therapy in those cases in which the rapid development of a high level of drug is imperative but to urge an increase in the trials with oral therapy in those infections in which it is not. It is believed that the results obtained in these studies especially warrant such trials with the solubilized preparation of amphotericin B.

CONCLUSIONS AND SUMMARY

A solubilized preparation of amphotericin B, designed for infusion into human beings, was orally administered to mice with experimental histoplasmosis, coccidioidomycosis, and cryptococcosis. Total dosages of as little as 70 mg./Kg. prolonged the survival of mice and produced negative cultures even when initiation of therapy was delayed as long as four and in some instances 10 days after infection. Serum levels of 1.2 µg./ml. were demonstrated after only four days of oral therapy with 25 mg./Kg., or a total dosage of 100 mg./Kg.

These results indicate that the oral administration of solubilized preparations warrants clinical trial in systemic mycotic infections in man.

ACKNOWLEDGMENT

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Clinical Results in the Treatment of American Leishmaniasis with Oral and Intravenous Amphotericin

TANCREDO A. FURTADO

*Dermatology Clinic, Faculty of Medicine, University of Minas Gerais and
National Institute of Rural Endemic Diseases, Research Center, Belo
Horizonte, Brasil*

Several drugs have been used in the treatment of American leishmaniasis, among which antimonial compounds, amino-arsenophenol, and di-aminodiphenoxipentane are considered the most effective.^{1,7,8} Usually, the cutaneous lesions respond to these drugs, whereas the oral-nasopharyngeal manifestations appear to be resistant. Thus, either the therapy is not satisfactory or clinical recurrences occur. Therefore, clinical investigation of new drugs in the treatment of American leishmaniasis, an endemic disease in extensive tropical rural regions, is entirely justified.

Although amphotericin* is primarily indicated in the treatment of deep mycosis caused by yeast and yeastlike fungi,^{5,10-12} evaluation of its action on *Leishmania brasiliensis* in vitro as well as in human disease was considered. Preliminary tests in culture tubes showed the high leishmanicidal action of amphotericin B in a concentration as low as 0.01 µg./ml. of culture media.³

The favorable clinical response obtained in the first 4 cases treated by Furtado,² confirmed by Lacaz et al.,⁶ led me to investigate the antibiotic further. In this paper the results observed in the treatment of 10 patients with American leishmaniasis are reported.

MATERIALS AND METHODS

Ten patients with American leishmaniasis (1 with the mucous form, 4 with the cutaneous form, and 5 with the mucocutaneous form) were treated with amphotericin. Nine patients were men and one was a woman. Age and color of the patients, duration of the disease, and a summarized description of the cutaneous and mucosal lesions are shown in table I. In every case the diagnosis was made on clinical and epidemiological grounds but was confirmed by a positive intradermal test, whose sensitivity and specificity are well demonstrated.^{4,9} The search for *L. brasiliensis* in smears and in histological sections was positive in only 4 cases. It should be stressed, however, that the number of organisms in the lesions is inversely proportional to the duration of the disease. Five patients had been previously treated with antimonial and/or arsenical drugs, with no results, and the remaining patients had not received any treatment.

The antibiotic was administered in two forms: amphotericin A/B tablets, containing 69.5 mg. of amphotericin A and 100.8 mg. of amphotericin B; and amphotericin B in intravenous drip, at a maximum daily dose of 50 mg./500 ml. of 5 per cent glucose in water, over a period of three to four hours. In table I are indicated

* The trade name of E. R. Squibb & Sons Division, Olin Mathieson Chemical Corp., for amphotericin is Fungizone. The amphotericin was kindly supplied by this company.

TABLE I

Clinical Results in the Treatment of American

Case no. Age, yr. Race	Diagnosis	Duration of the disease	Administration	
			Dosage and route	Period, days
1 46 W	Mucocutaneous leishmaniasis. Ulceration of nares; destruction of nasal cartilaginous septum; infiltration of uvula and pharynx	6 months	Oral: 3.2 Gm. daily for 90 days Intravenous: 50 mg. daily for 5 days	107
2 36 W	Cutaneous leishmaniasis. Vegetating and papular ulcerated lesions on the face, arms, legs, trunk and dorsum of feet	3 months	Intravenously, gradual increase from 12.5 to 50 mg. daily, with irregular lapses owing to venous irritation	72
3 48 N	Cutaneous leishmaniasis. Verrucous lesions of nose	6 months	Oral: 3.2 Gm. daily for 65 days Intravenous: 25 mg. daily for 4 days	73
4 6 W	Cutaneous leishmaniasis. Ulcerations of legs, abdomen and face	10 months	Oral: 1.8 Gm. daily for 45 days Intravenous: gradual increase from 12.5 to 25 mg. daily for 23 days. After a 30 day interval, 25 mg. intravenously, daily, for 19 days	125
5 46 W	Mucocutaneous leishmaniasis. Ulceration of angle of mouth; infiltration and ulcerative lesions of mouth, pharynx, and larynx	4 years	Oral: 3.2 Gm. daily for 22 days Intravenous: gradual increase from 12.5 to 50 mg. for 20 days. After a 30 day interval, 50 mg. intravenously, daily for 11 days	52
6 13 W	Cutaneous leishmaniasis. Ulceration of right elbow	4 years	Oral: 1.8 Gm. daily for 20 days Intravenous: gradual increase from 12.5 to 25 mg. daily for 21 days	41
7 38 N	Mucous leishmaniasis. Perforation of nasal septum; infiltration and vegetating lesions in the mucosa of mouth, larynx, and pharynx	5 years	Intravenously, gradual increase from 12.5 to 50 mg. daily	33
8 22 N	Mucocutaneous leishmaniasis. Vegetating lesion of nose; destruction of anterior cartilaginous nasal septum	7 months	Intravenously, gradual increase from 12.5 to 50 mg. daily	40
9 58 N	Mucocutaneous leishmaniasis. Ulcerations of lower lip; gangosa-like destruction of nose and middle part of upper lip; infiltrative lesions in mucosa of mouth and pharynx	15 years	Intravenously, gradual increase from 12.5 to 50 mg. daily	21
10 51 W	Mucocutaneous leishmaniasis. Ulcerations of nasal vestibulum and penis; infiltrative lesions of tongue and palatum	4 months	Intravenously, gradual increase from 12.5 to 50 mg. daily	30

the form of drug, dosage and route, duration of treatment, and total dosage for every patient.

In order to evaluate the tolerance to the drug, liver function tests, urinalyses, blood counts, and electrocardiograms were performed before and after therapy.

RESULTS AND COMMENTS

The results of treatment are presented in table I. Amphotericin A/B was initially tried due to ease of oral administration. This is of utmost importance in a disease such as American leishmaniasis that does not require hospitalization. In spite of

TABLE I

Leishmaniasis with Oral and Intravenous Amphotericin

Total dosage		Clinical results	Side effects	Remarks
Tablets, Gm.	Solution, mg.			
240	250	Marked improvement with administration of oral amphotericin; complete healing of lesions with only 250 mg. intravenously	Febrile reactions avoided by the use of aspirin before and during infusions	No relapse noted during a follow-up period of 10 months
	1500	Clinically effective. Complete healing of lesions	Febrile reactions avoided by the use of 1.50 Gm. of aspirin daily. Venous irritation	No relapse noted during a follow-up period of seven months
192	200	Fair clinical response to the administration of oral amphotericin; complete healing of lesions with only 200 mg. intravenously	Chills and fever with infusions not controllable with aspirin	Patient previously treated with antimonial preparation, unsuccessfully. No relapse noted during a follow-up period of four months
81	870	No clinical response to the administration of oral amphotericin; complete healing of lesions with the intravenous therapy	Slight febrile reaction with infusions	No relapse noted during a follow-up period of four months
67.2	1450	No clinical response to the therapy with oral amphotericin; complete healing of lesions with the intravenous therapy	Febrile reactions avoided by the administration of 1.50 Gm. of aspirin during infusions. Nausea and vomiting with infusions	Patient previously treated with 210 injections of antimonial preparations, unsuccessfully. No relapse noted during a follow-up period of three and a half months
36	312.5	Poor clinical response to the therapy with oral amphotericin; complete healing of lesions with the intravenous therapy	Slight febrile reaction, nausea and vomiting with infusions	Patient previously treated with antimonial preparation, unsuccessfully. No relapse noted during a follow-up period of four months
	900	Clinically effective. Complete healing of lesions	Febrile reactions avoided by the administration of 1.50 Gm. of aspirin during infusions. Anorexia and nausea with infusions. Venous irritation	Patient previously treated with antimonial and arsenical preparation, unsuccessfully. No relapse noted during a follow-up period of three months
	1525	Clinically effective. Complete healing of lesions	No evidence of toxicity noted. Slight venous irritation with infusions	No relapse noted during a follow-up period of three months
	700	Clinically effective. Complete healing of lesions	Febrile reactions avoided by the administration of 1.5 Gm. of aspirin during infusions. Nausea with infusions	Patient previously treated with antimonial preparation (antimonium potassium tartrate), unsuccessfully. No relapse noted during a follow-up period of three months
	1350	Clinically effective. Complete healing of lesions	Febrile reactions with infusions not controllable with aspirin. Nausea and vomiting with infusions	Patient still hospitalized, under observation

large doses used (3.2 Gm. daily for adults and 1.8 Gm. for children) only 2 patients were markedly improved, although they did not obtain complete involution of their lesions; 2 other patients did not show any response and 1 patient obtained slight improvement.

Amphotericin B, in intravenous drip, was subsequently given to the 5 patients who had taken oral amphotericin A/B and to 5 other patients, causing complete healing of the cutaneous as well as of the mucosal lesions in all of them. The 2 patients who showed marked improvement with oral amphotericin A/B required only total dosages of 250 and 200 mg., respectively. The patient who had slight improvement received a total dosage of 312.5 mg., although he was a child. In the remaining 7



FIG. 1 (top). Case 2 before treatment.

FIG. 2 (bottom). Case 2, showing complete healing of all lesions after 1500 mg. of amphotericin B.



FIG. 3 (left). Case 4 before treatment.

FIG. 4 (right). Case 4 after 870 mg. of amphotericin B, illustrating total healing of the cutaneous lesions.

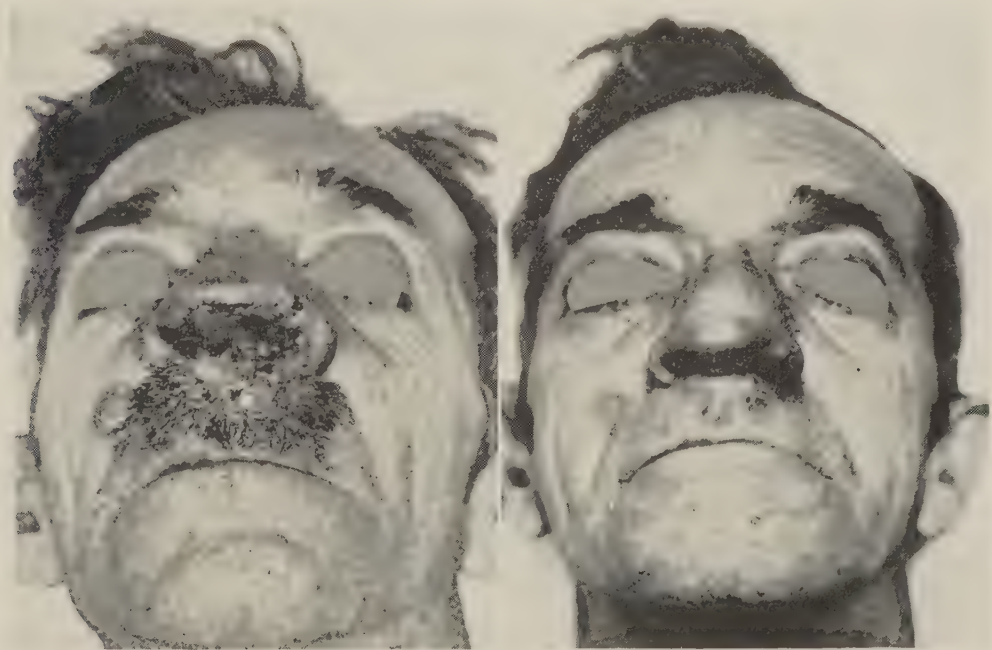


FIG. 5 (left). Case 10 before treatment.

FIG. 6 (right). Complete regression of the cutaneous and the mucosal lesions of tongue and palatum in Case 10, after 1350 mg. of amphotericin B.

patients, the total dosage ranged from 700 mg. to 1525 mg. and the maximum daily dose was 50 mg. (figs. 1-6).

The tendency to recurrence is well known in American leishmaniasis with the classical therapeutic methods, using antimonial and arsenical drugs. In the group of patients treated no sign of clinical relapse was noticed, although the follow-up period, ranging from 15 days to 10 months, cannot be considered long enough to allow for a definite conclusion on this topic.

In the reports on the use of amphotericin B in the therapy of deep fungous diseases febrile reaction during intravenous drip has frequently been mentioned. In the group of patients treated, the simultaneous administration of 1.5 Gm. of acetylsalicylic acid prevented the elevation of temperature in 5 patients or this was minimal in the remainder. Other undesirable side effects, such as nausea, vomiting, anorexia, malaise, joint pains, although never severe enough to require interruption of therapy, were observed in 6 patients. Venous irritation determined by prolonged injection was observed in 7 patients but not in the 3 patients who had received smaller total dosages. Laboratory tests before and after therapy revealed no renal, hepatic, or hematopoietic toxicity. The only abnormality was the disclosure of hypertension in 1 patient.

In conclusion, this investigation on the use of amphotericin B in the treatment of American leishmaniasis in 10 patients gave favorable results as evidenced by prompt healing of the cutaneous as well as the more resistant mucosal lesions.

SUMMARY

Ten patients with American leishmaniasis (1 with the mucous form, 4 with the cutaneous form, and 5 with the mucocutaneous form) were treated with amphotericin. Five patients (cases 3, 5, 6, 7, and 9) had been previously treated with antimonial and/or arsenical drugs, with no results, and the remaining patients had not received any treatment whatsoever.

Amphotericin A/B tablets were administered to 5 patients (cases 1, 3, 4, 5, and 6) with poor or no results, after total doses ranging from 36 to 240 Gm. Amphotericin B was administered to this group and to 5 additional patients, in intravenous drip, at a maximum daily dose of 50 mg. suspended in 500 ml. of aqueous 5 per cent dextrose solution, over a period of three to four hours. The total dose ranged from 200 to 1450 mg. No relapse was noted during follow-up periods ranging from 15 days to 10 months.

Results were excellent and complete healing of the cutaneous and mucous lesions was obtained in all cases. Good tolerance to the drug and absence of toxicity were evidenced by clinical observation of the patients and control laboratory studies (liver function tests, urinalysis, blood count, and electrocardiogram). Febrile reactions and slight venous irritation were the only side effects observed.

The present investigation allows for the conclusion that amphotericin B is effective in the treatment of American leishmaniasis.

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Clinical Experiences with Amphotericin B

GERALD L. BAUM AND JAN SCHWARZ

*Veterans Administration Hospital, University of Cincinnati College of Medicine,
and Jewish Hospital, Cincinnati, Ohio*

Until recently, the therapy of the deep fungus diseases has been most ineffective. A variety of agents have been used, but in cases of blastomycosis, histoplasmosis, and cryptococcosis the results were all poor, especially in disseminated disease.

In the late 1940's and early 1950's, the advent of the aromatic diamidines²⁰ improved the picture considerably. These agents proved to be the first effective therapeutic agents in the treatment of North American blastomycosis,^{14, 20} though their use was attendant with many serious side effects. More recently, cases treated with stilbamidines have manifest recurrence.⁶

Within the last five years amphotericin B has been found to be effective against a variety of the deep fungi, both in vitro and in vivo.^{11, 18, 21, 23} Experimental studies in animals have emphasized the effectiveness of this drug against a variety of deep fungus diseases.^{2, 3, 8, 16}

Clinical experience has been accumulating to corroborate the early impressions.^{1, 4, 5, 7, 9, 10, 12, 15, 17-19, 21, 24} At the present time amphotericin B is considered by many to be the treatment of choice in North American blastomycosis, histoplasmosis, and cryptococcosis.

The present experience deals with 13 cases of deep fungus diseases treated with amphotericin B.

There were 6 cases of histoplasmosis, 4 of which were acute and 2 chronic (table I). The ages of the patients with acute disease ranged from 2 1/3 to 41 years; the 2 chronic patients were 67 and 68 years old. All patients were Caucasian men. Of interest is the fact that patient C. K. had the symptoms and roentgenographic appearance of a typical "epidemic" case following exposure to dust in a chicken house, in 1956. This appeared to clear roentgenographically, but he had a reactivation in the fall of 1958. It was at this time that a lung biopsy proved the diagnosis, and the patient received amphotericin B therapy. He initially got worse after therapy began but then improved and has been well for at least three months' post-therapy. The chronic patient who died was originally thought to have a bronchogenic carcinoma of the right upper lobe, and at surgery the diagnosis of histoplasmosis was made by biopsy. Despite his initial good response to amphotericin B therapy, a new mass developed in the lower right lung. There was general progression of symptoms, and the patient died. At autopsy cancer was present, but no active histoplasmosis was found.

The 5 patients with blastomycosis varied in age from 33 to 92 years (table II). All were men—2 Negro and 3 Caucasian. Patient A. S. was of particular interest because the *Blastomyces* was found in a culture of the synovial fluid from the right knee. The only other symptoms the patient manifested were related to degenerative vascular disease of the brain, from which he ultimately died. His knee had cleared completely under amphotericin B therapy. This was the only patient in

TABLE I

Histoplasmosis Cases Treated with Amphotericin B

Patient	Age, yr.	Race*	Sex	Diagnosis	Treatment	Result	Comment
C. K.	19	C	M	Acute diffuse pulmonary	Total dose, 1.425 Gm. intravenously; plus sodium sulfadiazine	Slight roentgen-ray clearing; clinically well for three months	Reactivation two years after initial disease
L. T.	28	C	M	Acute diffuse pulmonary	Total dose, 0.285 Gm. intravenously; plus sodium sulfadiazine	Clinically well for 18 months (no roentgenogram)	
L. T. Jr.	2 1/3	C	M	Acute diffuse pulmonary	Total dose, 0.189 Gm. intravenously; plus sodium sulfadiazine	Clinically well for 18 months (no roentgenogram)	
W. P.	41	C	M	Acute focal pulmonary	Total dose, 3.378 Gm. intravenously; plus sodium sulfadiazine	Roentgen-ray cleared 80 per cent; sputum became negative; symptoms cleared 100 per cent	
J. O.	67	C	M	Chronic pulmonary	Total dose, 1.275 Gm. intravenously; plus sodium sulfadiazine, total dose, 68.0 Gm. orally	Roentgen-ray cleared 20 per cent, but lesion worse 6 months later; symptoms improved but got worse six months later; died	Death due to carcinoma lung; no active histoplasmosis at postmortem examination
I. M.	68	C	M	Chronic pulmonary with cavitation	Total dose, 0.870 Gm. intravenously; plus buffer	Roentgen-ray unchanged; sputum culture negative x 6 after 8 positive cultures; symptomatic improvement after three months was 90 per cent	

* C = Caucasian.

whom oral amphotericin was used exclusively. Two other patients received oral amphotericin B therapy following intravenous therapy.

The 2 cryptococcosis cases treated with amphotericin B were Caucasian men, aged 32 and 55 years (table III). One had a typical case of meningitis in which intensive therapy produced complete clearing of the spinal fluid both by culture and analysis. The other case was an unusual one of isolated pulmonary nodules proved by lung biopsy to be due to *Cryptococcus neoformans*. Following prolonged therapy, complete roentgen-ray clearing occurred.

TABLE II

Blastomycosis Cases Treated with Amphotericin B

Patient	Age, yr.	Race*	Sex	Diagnosis	Treatment	Result	Comment
J. E.	77	N	M	Pulmonary and cutaneous	Total dose, 1.651 Gm. intravenously	Roentgen-ray clearing 80 per cent; skin clearing 100 per cent; follow-up 26 months	
J. N.	84	C	M	Pulmonary and cutaneous	Total dose, 0.615 Gm. intravenously	Roentgen-ray clearing 60 per cent; skin clearing 100 per cent; follow-up three months	
D. T.	35	C	M	Pulmonary and cutaneous	Total dose, 1.700 Gm. intravenously; total dose, 60.0 Gm. orally	Roentgen-ray clearing 60 per cent; skin clearing 100 per cent; follow-up one month	Previous failure after three courses of aromatic diamidines
A. S.	92	C	M	Right knee, acute synovitis	Total dose, 416.0 Gm. orally	Complete clearing of knee; died	Pulmonary cavity seen at postmortem examination
E. A.	33	N	M	Cervical adenopathy hilar adenopathy pneumonia	Total dose, 1.804 Gm. intravenously	Roentgen-ray clear; lymph nodes unchanged; follow-up two months	

* C = Caucasian; N = Negro.

TABLE III

Cryptococcosis Cases Treated with Amphotericin B

Patient	Age, yr.	Race*	Sex	Diagnosis	Treatment	Result	Comment
S. K.	32	C	M	Nodular pulmonary lesions	Total dose, 2.035 Gm. intravenously; plus sodium sulfadiazine	Roentgen-ray clearing 100 per cent; symptom clearing 100 per cent; follow-up eight months	
W. B.	55	C	M	Meningitis	Total dose, 3.70 Gm. intravenously; plus sodium sulfadiazine	Cerebrospinal fluid clearing by count, analysis, culture 100 per cent; follow-up one month	

* C = Caucasian.

RESULTS

Of the 4 acute histoplasmosis cases, all have recovered (with a maximum follow-up of 18 months). Of the 2 chronic cases of histoplasmosis, one died due to carcinoma of the lung and the other remains clinically well three months after the end of therapy. Of interest is the fact that though sputum cultures are now negative for *Histoplasma capsulatum*, the chest roentgenogram has been unchanged by therapy. Of the 5 patients with blastomycosis, 4 are well, with a maximum follow-up of 26 months; 1 died, apparently of vascular disease unrelated to blastomycosis. Of the 2 patients with cryptococcosis, both are well, with one month and eight month follow-ups available.

DOSAGE

The dose of amphotericin B in our cases ranged from 0.5 to 1.0 mg./Kg. This was given intravenously in 500 to 1000 ml. 5 per cent glucose in water over a four to six hour period. The frequency of therapy varied from daily to three doses per week and was controlled by the advent of severe toxic reactions. The duration of therapy varied from 11 days (patient L. T.) to six months (patient C. K.). The infusion was prepared fresh for each injection in all patients by suspending the amphotericin powder in 15 to 20 ml. of 5 per cent glucose in water and withdrawing the appropriate dose and adding it to the infusion bottle.

An attempt to prevent the toxic effects by raising the pH of the infusion has been suggested.¹³ Buffer solution was used in 1 patient (I. M.) without any ameliorating effect. Sodium sulfadiazine was used in 7 patients for this same reason and because of suggestion that it increased the effectiveness of amphotericin B against experimental histoplasmosis. No effect was noted.

TOXICITY

Of the 13 patients treated with amphotericin B, all but the 2 $\frac{1}{3}$ year old child manifested symptoms of toxicity due to amphotericin B. The commonest symptoms were as reported by other authors—nausea, vomiting, anorexia, fever, chills, rising blood urea nitrogen, and thrombophlebitis. In at least one instance treatment was discontinued by the patient because of side effects long before an optimum course was given (patient L. T.). In one other case treatment was curtailed after three

months because of persistence of a high blood urea nitrogen (patient I. M.). In several other cases symptoms were so severe that treatment had to be delayed several times during the course (patients S. K., J. O., C. K., and W. B.). The relative frequency of these symptoms in our series is noted in table IV.

DISCUSSION

Amphotericin B has proved effective in the test tube and in the experimental animal against *Blastomyces dermatitidis*, *H. capsulatum*, and *C. neoformans*.^{2, 3, 8, 11, 16, 18, 21-23} The results of clinical experience in the literature indicate that this drug is the treatment of choice in North American blastomycosis, histoplasmosis, and cryptococcosis.^{1, 4, 5, 7, 9, 10, 12, 15, 17-19, 21, 24}

One of the problems of evaluating the effects of this drug in these diseases is the fact that all of them may have prolonged phases of arrest. North American blastomycosis may go on for years without progression, provided there is no activity of the primary pulmonary focus. Histoplasmosis is benign in the overwhelming majority of cases. Cryptococcosis is known to have a variable course with long periods of clinical inactivity. For this reason, clinical evaluation of results of amphotericin B therapy must be viewed with some reservation, and long-term follow-up is mandatory.

In our experience amphotericin B is most dramatic in its effect on North American blastomycosis. In 4 of the 5 patients treated active pulmonary lesions were present. In each of them clinical and roentgen-ray response was noted with amphotericin B therapy. Of particular note is the fact that there has been no recurrence in any of the cases so treated. Of additional interest in this regard is that 1 patient (D. T.) had had repeated courses of the aromatic diamidines with recurrence after each. Following amphotericin B therapy the patient has had no recurrence, and the degree of clearing is greater than after the aromatic diamidines.

Histoplasmosis is somewhat more difficult to evaluate from a therapeutic standpoint. The 4 acute cases treated in this series may well have responded without therapy. The 2 1/3 year old child was probably in the greatest danger of active progressive dissemination. By the time he was treated most evidence of acute illness had passed. However, it was felt that because of his youth and because of the widespread nature of his lesions the protection of amphotericin B was due him. Patient C. K. has done very well following therapy with amphotericin B; however, it is felt that long-term follow-up of this case is extremely important in order to determine

TABLE IV
Toxic Effects from the Use of Amphotericin B

Toxic effect	No. of cases
Nausea	8
Anorexia	7
Chills and fever	7
Blood urea nitrogen elevation	6 (highest blood urea nitrogen elevation to 56 mg. per cent)
Emesis	3
Superficial phlebitis	3

final effect of the drug, since reactivation was evident two years after initial clinical disease.

Admittedly 2 cases of cryptococcosis are inadequate to evaluate the effect of this drug in this particular disease. However, the completeness with which the active pulmonary lesions cleared in patient S. K. was very striking. In addition, the prompt clearing in patient W. B. of the spinal fluid, from which positive cultures were obtained with ease prior to treatment, was remarkable. We therefore feel that a strong hint of clinical effectiveness is given by these cases.

Despite the fact that toxic symptoms were almost universally present, there is no evidence in any case treated in our experience of lasting effect after amphotericin B was discontinued. We therefore feel that though delay in treatment and diminution of dosage may be indicated by extreme symptomatology, permanent discontinuation of therapy is not warranted.

Variation in dose of the drug from 0.5 to 1.0 mg./Kg. seems indicated according to the patient's tolerance. Frequency of administration is dependent also on degree of toxicity. Length of treatment and total dose are unknown and will depend on long-term follow-up of treated cases. Intravenous therapy is the most effective, though oral dosage is tempting because of the absence of side effects. Despite clinical response in the 1 patient treated with oral amphotericin alone, we are skeptical of its use in a truly urgent or emergency situation. Blood levels were not obtainable after administration of as much as 4 Gm./day in normal subjects.

SUMMARY

Clinical experiences with amphotericin B in the treatment of 6 cases of histoplasmosis, 5 cases of North American blastomycosis, and 2 cases of cryptococcosis are reported.

An over-all mortality of 1 of 6 histoplasmosis cases and 1 of 5 blastomycosis cases with no deaths in the cryptococcosis cases is noted.

Evidence of clinical effectiveness in North American blastomycosis, histoplasmosis, and cryptococcosis is definitely inferred.

The toxicity of these drugs, though annoying, has not as yet proved serious.

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Treatment of Cutaneous Candidiasis and Pyodermas with Anti-infectives and Triamcinolone Acetonide in a Single Topical Preparation

C. M. HOWELL, JR.

*Department of Medicine, Bowman Gray School of Medicine,
Wake Forest College, Winston-Salem, N. C.*

The successful specific treatment of common skin infections by surface application of highly active antimicrobial agents and the topical use of corticosteroids to control accompanying inflammation and pruritus represent material advances in dermatological therapy. Properly applied to the skin, antibiotics attain a local concentration substantially in excess of the maximum achieved in other tissues and usually greater than that necessary to inhibit the proliferation of most bacteria responsible for primary or superimposed pyodermas. The discovery and isolation of nystatin has made available a practical agent for combatting candidiasis as a primary or complicating skin problem. Since many of the prevalent dermatological disorders are initiated or complicated by bacterial and/or fungal invaders, the use of nystatin in combination with broad-spectrum antibacterial drugs provides a more complete approach to the treatment of infected skin lesions.

Precise diagnosis of all cutaneous infections on the basis of clinical characteristics or through bacteriological procedures is desirable but not always practicable. Where infection is the primary cause of skin disturbance, distinguishing features are more readily detected. Secondary infections, however, are usually nondescript, with structure and form being dependent on the nature of the underlying dermatosis and involvement varying from a simple contamination to an extensive prurulent process. Isolation and sensitivity testing of the causative microorganisms can be a tedious and unrewarding operation, which merely delays the initiation of treatment. Although the physician is not relieved of the responsibility for carrying out such procedures, it is recognized that in vitro sensitivity tests are not always guides to the clinical efficacy of antibiotic agents. Lack of susceptibility demonstrated in the laboratory is not an absolute indication of resistance to concentrations achieved on the skin surface.

While microbial inhabitants of the skin are many and constantly subject to change, experience has implicated only a few as causative agents in common skin infections. Staphylococci, streptococci, and, to a lesser degree, *Pseudomonas*, coliform organisms, and *Proteus* species are the usual bacteria involved in primary or secondary pyodermas. Apparently, changes induced in bacterial flora by the use of broad-spectrum antibiotics has increased the prevalence of *Candida albicans* and the incidence of cutaneous candidiasis. Secondary fungus infection of various dermatoses is usually caused by *C. albicans* and *Candida* are also commonly responsible for primary infection of intertriginous areas.

Combinations of a topically active corticoid and broad-spectrum antibiotics are now widely employed for full control of many dermatological conditions where bacterial infection is an existing or threatening factor.¹⁻⁶ In a high percentage of

TABLE I

Incidence of Candidiasis and Pyoderma as Primary or Superimposed Cutaneous Disorders and Results of Treatment

Primary skin disorder	Total no. patients	Nature of infection		Response			
		Candidiasis	Pyoderma	Excellent	Good	Fair	Poor
Candidiasis	57	57	17	44	11	2	0
Contact dermatitis	4	4	2	3	0	1	0
Dermatitis herpetiformis	1	1	0	0	0	0	1
Eczematoid dermatitis, chronic	16	16	12	13	3	0	0
Lichen simplex chronicus	1	1	1	0	1	0	0
Neurodermatitis	7	7	1	4	2	1	0
Pemphigus vulgaris	1	1	0	1	0	0	0
Psoriasis	2	2	1	1	1	0	0
Verruca acuminata	1	1	1	1	0	0	0
Total	90	90	35	67 (74.5%)	18 (20.0%)	4 (4.4%)	1 (1.1%)

cases, these preparations have been found to relieve pruritus and resolve inflammation promptly, to suppress infectious agents effectively, and to promote the early clearance of the underlying cutaneous eruption. The addition of nystatin to this type of formulation extends the usefulness of topical treatment to primary and superimposed outbreaks of candidiasis of the skin by providing the drug with pronounced activity against *C. albicans*.⁷⁻⁹ The specific preparation considered in this communication (Mycolog*) contains 1.0 mg. triamcinolone acetonide plus 2.5 mg. neomycin, 0.25 mg. gramicidin, and 100,000 units nystatin per Gm. of oleaginous ointment base. It appears a promising medication for the management of both primary and secondary pyodermas and *Candida* infections.

MATERIALS AND METHODS

The Medication. In dermatological practice, the corticoid component of the preparation studied, triamcinolone acetonide, in 0.1 per cent concentration has been reported therapeutically superior to 1.0 per cent hydrocortisone, the prototype of all topical corticosteroid anti-inflammatory agents.¹⁰⁻¹³ Neomycin and gramicidin fulfill the accepted requirements for antibacterial drugs to be employed for local therapy: They are effective against the common offending organisms; they are non-irritating and seldom cause sensitization reactions; they are stable and can be incorporated in cosmetically acceptable vehicles; and they are unlikely to be used in the treatment of systemic infections. It has also been suggested that under certain conditions the concomitant use of these two antibiotics may minimize the emergence of resistant strains.¹⁴ Nystatin is the preferred agent for combatting candidiasis.

Selection of Patients. A series of 58 female and 32 male patients—13 children and 77 adults—ranging in age from 17 months to 65 years were selected for treatment because of an overt pyodermatous eruption or a proved diagnosis of

* The trade name of E. R. Squibb & Sons Division, Olin Mathieson Chemical Corp., for a combination of triamcinolone acetonide, neomycin, gramicidin, and nystatin is Mycolog. The drug was supplied for this study through the courtesy of Dr. J. T. Groel, of E. R. Squibb & Sons.

TABLE II

Duration of Primary or Superimposed Cutaneous Infection and Results of Treatment

Duration of infection	Number of patients	Duration of treatment, days	Response			
			Excellent	Good	Fair	Poor
Less than 4 weeks	19	7-21	15	4	0	0
4-11 weeks	23	9-20	19	3	1	0
3-6 months	23	13-26	18	4	1	0
7 months-1 year	13	11-23	9	4	0	0
More than 1 year	10	15-23	4	3	2	1
Unknown	2	8-19	2	0	0	0
Total	90		67 (74.5%)	18 (20.0%)	4 (4.4%)	1 (1.1%)

candidiasis. A history of cutaneous eruption of five days to one year in duration was obtained in most instances, although 12 patients experienced alternate episodes of remission and flare-up for periods extending from 2 to 28 years. From clinical findings, mycological involvement was suspected in all of these cases, and characteristic filaments and spores of *C. albicans* were subsequently demonstrated by direct examination of skin scrapings cleared in 10 per cent potassium hydroxide and by culture of cutaneous material. The incidence of candidiasis and pyoderma is reviewed in table I. In 57 patients, *C. albicans* was the primary etiological agent responsible for a pruritic patchy eruption at the usual sites of predilection—the intertriginous folds, the perianal region, and the vulva—or for an angular stomatitis. In 33 cases, pre-existing lesions were infected by the fungus. A secondary pyoderma was present in 35 of the total group of patients.

Treatment. Patients selected for treatment were instructed to discontinue all previous medication and to apply the ointment twice daily to affected areas, gently and thoroughly rubbing it into the skin. Treatment of oozing and weeping lesions was initiated with compresses or soaks of potassium permanganate (1:8000) until exudation subsided, after which the usual ointment applications were carried out. Occasionally, compresses or soaks of boric acid or saline were employed concomitantly and some patients were given a hypoallergenic cake for cleansing the skin. Several women with eruptions covering the groin and vulval regions were furnished with nystatin vaginal tablets to be used in conjunction with the ointment for control of vaginitis. Attention was given to the patients' general physical status, and rarely, ataraxics, antihistamines, or crude liver injections were given as indicated. Treatment was continued for 7 to 26 days, depending on clinical response.

Previous Medication. As might be expected with any dermatological group, self-medication with "home-type" remedies and previous application of other prescribed topical preparations were common. Only 12 of the 90 patients in this series had not been exposed to any form of therapy prior to the present use of the ointment. While many of the agents could not be identified by the patient, direct application of corticosteroids, gentian violet, potassium permanganate, and iodochlorhydroxyquin appeared to be a frequent practice. All were discontinued.

RESULTS

The results of treatment in relation to the underlying pathology and duration of candidiasis and bacterial infection are presented in tables I and II. Early subsidence of the active infectious process, control of itching and inflammation, and progressive involution of the underlying eruption was observed in 67 (74.5 per cent) of the patients treated and these were considered as showing an excellent response to medication. Good results were accomplished in 18 (20 per cent) patients, with improvement being somewhat slower in this group. Very gradual benefits were observed in 4 cases and only 1 patient was considered to respond poorly to medication. In this instance, although the secondary infection cleared, dermatitis persisted, and subsequently, a diagnosis of dermatitis herpetiformis was established on the basis of clinical observations and the results of biopsy. The high percentage of good to excellent results is attributed to an impressive complement of antifungal, antibacterial, and anti-inflammatory agents none of which interfered with the action of any of the others and, in part, to the fact that medication was generally well accepted and patient cooperation in carrying out the prescribed regimen was at a maximum.

A noteworthy observation in this series was the absence of recurrent episodes of bacterial or fungal infection. Along with the control of *Candida* and/or bacterial superinfection, clearing of the underlying skin disorder occurred promptly in self-limited contact dermatitis and with reasonable speed in eczematoid dermatitis and in acute and chronic forms of neurodermatitis. The patient with verruca acuminata showed a rapid and complete response to treatment. Some degree of improvement was achieved in the 2 cases of psoriasis for as long as medication was continued. The 1 case of pemphigus vulgaris was effectively controlled with systemic administration of triamcinolone, while the ointment was simultaneously applied to infected lesions. In none of the cases reported here was there any evidence of primary irritation or of sensitivity reactions to the ointment.

COMMENT

Direct local attack to suppress infection and inflammatory changes in the skin is, in principle, most effectively accomplished with a topical preparation combining potent antimicrobials with a corticosteroid. Translated into clinical terms, gratifying results were obtained in a high percentage of cases of cutaneous candidiasis and of pyoderma treated with triamcinolone acetate, neomycin, gramicidin, and nystatin in an ointment vehicle. Where candidiasis is suspected as a primary or superimposed condition, specific diagnosis is secured by demonstration of *C. albicans* in skin scrapings and in cultures of cutaneous material, and nystatin provides the most satisfactory antifungal action. On the other hand, a number of restrictions precisely define the antibacterial drugs that may be employed locally in superficial infections. These agents must be unlikely prospects for use in systemic infection. They must be stable in the vehicle employed, nonirritating, and must seldom cause sensitization reactions. In particular, they must be effective against the common offending organisms, the staphylococci, streptococci, *Pseudomonas*, coliforms, and *Proteus* species most often responsible for cutaneous infection. Gramicidin and neomycin

together continue to fulfill these specifications as well as any newer substances that may be available. Triamcinolone acetonide has been found therapeutically superior to hydrocortisone when applied to the skin in one tenth the concentration. These agents in combination provide a full complement of anti-inflammatory, antibacterial, and antifungal activity for treatment of infected dermatoses.

SUMMARY

Topical application of an ointment containing triamcinolone acetonide, neomycin, gramicidin, and nystatin for treatment of cutaneous candidiasis and pyoderma, whether primary or secondary to other skin pathology, resulted in an early subsidence of the active infectious process, suppression of itching and inflammation, and progressive involution of the underlying eruption. Clinical response was graded as excellent in 67 (74.5 per cent) of the total series of 90 patients, good in 18 (20.0 per cent), fair in 4 (4.4 per cent), and poor in 1 (1.1 per cent). Control of infection was permanent, as far as could be determined, and there were no recurrences during this study. No evidence of primary irritation or of sensitivity reactions was observed, and in general, the medication was well accepted and the patient cooperation required for a successful outcome was obtained. Criteria for combining therapeutic agents in a comprehensive approach to the topical management of infected dermatoses are discussed.

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One Hundred Eight Cases of Pyogenic Dermatitis Treated with a Combination of Oxytetracycline and Oleandomycin

SYLVIO FRAGA AND EVERARDO MARQUES DOS SANTOS

Dermatologists 26th Ward, Santa Casa da Misericordia, Rio de Janeiro, Brasil

The purpose of this presentation is to evaluate clinically the effects of the combination of oxytetracycline and oleandomycin, given orally to patients with skin diseases of the pyoderma group.

The use of antibiotics in medicine has been a matter of numerous publications in the medical literature during the past decade.^{4, 7, 8, 10, 13} It is generally agreed that the great number of undesirable side effects and the development of resistant strains of bacteria are the main difficulties in treating the pyoderma group of dermatitis.

MATERIAL AND METHOD

All the cases presented had dermatitis due to bacteria. These were classified as furunculosis, 53 cases; erysipelas, 16 cases; ecthyma, 14 cases; perionyx, 4 cases; tropical ulcer, 7 cases; suppurative hidradenitis, 8 cases; cellulitis, 6 cases. These cases were diagnosed either clinically or by laboratory methods. The 108 cases selected had not received previous treatment. Each patient received a daily dosage of 1 Gm. of the combination oxytetracycline-oleandomycin divided into four doses of 250 mg. administered orally every six hours. The total dosage ranged from 3 to 19 Gm. (3 to 19 days). No topical medication was employed except for the hygienic measures used in the ulcerated lesions.

The patients were seen every four days, and the medication was discontinued after the signs of infection had subsided. The patients were observed until the complete cicatrization of the lesions.

In accordance with their intensity, the cases were divided into three groups: Slight (+); moderate (++); and severe (+++). Final evaluation of the results was represented as cure; improvement; no change.

RESULTS

The results obtained and the total amount of antibiotics used are shown in table I. Only 2 patients had the medication discontinued due to severe allergic reactions. The large tropical ulcers showed signs of improvement after 96 hours, while some cases of furunculosis had cleared after 12 hours.

DISCUSSION

Undesirable side effects were seen in 10 of 108 patients who received the antibiotics during a period of time that varied from 3 to 19 days (3 to 19 Gm.). Allergic dermatitis (generalized erythematous papulovesicular eruption) was observed in

TABLE I

Therapeutic Results of Treatment of Dermatoses

Disease	No. cases	Slight	Moderate	Severe	Minimum dose		Maximum dose		Cure	Improve-ment	Discon- tinued
					Gm.	Days	Gm.	Days			
Furunculosis	53	12	12	19	3	3	19	19	47	5	1*
Hidradenitis	8	2	4	2	6	6	10	10	7	1	—
Erysipelas	16	—	—	—	3	3	8	8	15	1	—
Ecthyma	14	5	7	2	3	3	10	10	13	—	1*
Perionyx	4	1	3	—	5	5	8	8	4	—	—
Tropical ulcer	7	3	2	2	8	8	15	15	6	1	—
Cellulitis	6	1	4	1	4	4	6	6	6	—	—

* Presented extensive allergic dermatosis which prevented continuation of the medication on the fourth day.

2 patients. This prevented continuation of the treatment. The eruption cleared after withdrawing the medication. Minor reactions, such as nausea, anorexia, epigastric complaints, and increase of bowel movements, were present in 8 patients. None of our patients presented perianal dermatitis or moniliasis of the upper respiratory and digestive tracts.

In our series, the widespread lesions, such as furunculosis, responded more favorably than the larger destructive processes as tropical ulcer. We assume that the size of the lesion (the amount of tissue destruction) is directly proportional to the duration and intensity of the treatment (total dosage). This assumption provides a more accurate evaluation of the treatment employed. We attributed our low incidence of untoward reactions to the low dosage and the short duration of the administration of the drug.

SUMMARY AND CONCLUSIONS

In our series of cases, we obtained a high percentage of effective results, consistent with the current literature. For practical purposes, the combination of oxytetracycline-oleandomycin should be considered as a new antibiotic entity.

In conclusion we would like to point out that the use of smaller quantities of these two antibiotics furnishes a better tolerance, a broader spectrum of action, and reduces the development of resistant strains of bacteria. In this study we were unable to prove a possible synergistic action. According to our observation, there was no need for increased dosage although it is possible to extend the treatment for a longer period of time and still have low incidence of undesirable side effects.

ACKNOWLEDGMENT

Dr. Jose Lisboa Miranda performed the laboratory tests. The Medical Department of Pfizer Corporation of Brasil provided the antibiotic employed in this study.

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Triacetyloleandomycin: Further Observations on the Treatment of Acne and Pyodermas

SIDNEY OLANSKY AND GLENN E. MCCORMICK, JR.

*Department of Medicine, Division of Dermatology,
Duke University Medical Center, Durham, N. C.*

Last year we reported¹ the use of triacetyloleandomycin* in the treatment of 40 cases of pustular acne and 21 pyodermas caused by *Staphylococcus aureus* resistant to one or more antibiotics. From these studies it was apparent that triacetyloleandomycin was effective in controlling the pustular lesions of acne, effective in managing pyodermas caused by *Staph. aureus* resistant to other antibiotics, and remarkably free of adverse reactions.

Some antibiotics, notably the tetracyclines and erythromycin, have shown encouraging results in controlling the pustular stages of acne. Few studies on the use of triacetyloleandomycin in acne have been reported. Leming et al² listed only 3 cases of pustular acne in their series of 100 patients. Frank and Stritzler³ treated 238 acne patients with oleandomycin or its triacetyl ester for 6 to 24 months on a daily dosage of 250 mg. They confirmed our results, since patients were very responsive to treatment, the relapse rate was high, and the presence of resistant *Staphylococcus* strains did not diminish the clinical response.

On the other hand, several investigators have noted the utility of triacetyloleandomycin in treating pyodermas. Albright and Hall⁴ reported 5 cases of furunculosis with four cures and one undetermined result. Isenberg and Karelitz⁵ mentioned good results in 15 cases of familial furunculosis. Of 4 nonfamilial cases, 2 responded well, 1 slowly, and 1 failed to respond. Three patients, who had recurrence more than two months after successful therapy, improved promptly again on triacetyloleandomycin. Mellman et al⁶ observed excellent clinical responses in three pyodermas. The Leming et al² series included 23 pyodermas with results classified as excellent in 19, good in 4. Excellent clinical results were recorded by Wennersten⁷ in 15 cases. Shubin et al⁸ treated 6 staphylococcal skin infections with rapid clinical improvement.

Our own series has been amplified during the past year by 92 additional pustular and cystic acne cases, 57 more pyodermas, and 3 miscellaneous infections.

SELECTION OF PATIENTS

Acne patients were placed on triacetyloleandomycin therapy if they had lesions that did not respond to routine diet, acne surgery, ultraviolet light, and local sulfur resorcinol preparations. The antibiotic was added to the previous regimen. In this series a bacterial vaccine was also used in 19 pustular and in 16 cystic cases. The antibiotic without vaccine was used in 41 of the 57 pustular acne cases, and in 16 of 35 with cystic lesions.

A coagulase-positive *Staph. aureus* was the etiological agent in 39 of the skin

* The trade name of Wyeth Laboratories for triacetyloleandomycin is Cyclamycin.

infections. Beta-hemolytic streptococci were recovered in 4 patients. Both staphylococci and streptococci were isolated in 1 case of pyoderma. *Pseudomonas* and *Escherichia coli* were recovered in an ulcer case, and in 13 patients no organism was isolated.

CASE DATA, METHODS, AND DOSAGE

The age range in the acne patients was 10 to 26 years, with half the patients between 18 and 21 years inclusive. Fifty-three of the 92 patients were boys. The majority were treated with 250 mg. triacetyloleandomycin twice a day for one or two weeks and then maintained on 250 or 125 mg. daily. Seventy patients were treated for one year, most of the others for six to nine months.

The bacterial vaccine was prepared from several strains of *Corynebacterium acnes* and *Staphylococcus albus* grown anaerobically. In the severe cases (showing cystic tendency) 0.1 ml. of a 1:100,000 dilution was given subcutaneously, increasing the volume by 0.1 ml. at each injection three times a week. When the volume reached 0.9 ml., the next injection was 0.1 ml. of a 1:10,000 dilution—and so on. When the concentration of vaccine became 1:250, patients were placed on a maintenance dosage of 0.5 ml. once a week for 10 weeks.

The pyodermas were treated with 250 mg. triacetyloleandomycin four times daily until the infection was controlled. This required four to seven days in 52 cases. Four patients received the drug for 14 days. In 1 stubborn case, 1 Gm. was given daily for one month and then 0.5 Gm. daily for another month.

RESULTS IN ACNE

The clinical results observed in the 57 cases of pustular acne and in the 35 cases of cystic acne are shown in table I. Results were classified as excellent if lesions healed, good if improvement was marked but healing incomplete, and fair if there was some improvement but pustular or cystic elements persisted.

TABLE I
Results of Triacetyloleandomycin Therapy in Acne

Diagnosis	No. cases	Results		
		Excellent	Good	Fair
Pustular acne				
Antibiotic therapy	41	19	20	2
Antibiotic plus vaccine	16	9	6	1
	57			
Cystic acne*				
Antibiotic therapy	16	7	9	0
Antibiotic plus vaccine	19	8	11	0
	35			

* One case was complicated by severe nephritis. The patient received 1 Gm. triacetyloleandomycin daily for one month, then 0.5 Gm. daily for 11 months. The patient also received 8 mg. triamcinolone daily, and responded very well with no side effects.

Triamcinolone was also used for another patient given the antibiotic, 0.5 Gm. daily for 12 months.

TABLE II

Results of Triacetyloleandomycin Therapy in Pyodermas

Diagnosis	No. cases	Results		
		Excellent	Good	Fair
Pyoderma	17	17	—	—
Furunculosis	11	9	1*	1†
Folliculitis	5	5	—	—
Impetigo	5	5	—	—
Infected contact dermatitis	3	3	—	—
Infected cyst	3	1	2	—
Cellulitis	2	1	1	—
Ecthyma	2	2	—	—
Infected eczema	2	2	—	—
Pustular bacterid	2	1	1	—
Infected finger, ulcers,‡ pustular eruption of face, pustular eruption of hands and feet, scleredema§	5	1	4	—
Total	57	47	9	1

* Furunculosis of scalp, nonhemolytic *Staphylococcus* resistant to tetracyclines.

† Furunculosis of axilla—hemolytic *Staphylococcus* resistant to penicillin, tetracyclines, and erythromycin.

‡ Ulcers of foot and leg—*Pseudomonas* and *E. coli*—good clinical result despite in vitro resistance to many antibiotics.

§ Received triamcinolone also.

Combining our 57 cases of pustular acne reported here with the 40 reported last year, we have a total of 97 patients, of whom excellent clinical results were obtained in 55 per cent and good results in another 36 per cent. Our first result category improved considerably, only 3 of 57 as compared with 6 of 40 a year ago. Expressed differently, triacetyloleandomycin nearly always cleared up the pustular lesions.

From table I it would appear that the vaccine had little influence on cystic acne. However, careful follow-up revealed that vaccine desensitization changed relapsing lesions months later—they were smaller, showed little induration, and almost no tendency to develop cysts. We plan to try placebo vaccine therapy to be sure these changes are not on a psychosomatic basis.

Twelve acne relapses were treated with erythromycin propionate* in the same dosage that had been effective with triacetyloleandomycin. All 12 patients responded poorly and asked to be replaced on the original antibiotic. Another 12 patients were treated similarly with tetracycline.† Six of these asked for resumption of triacetyloleandomycin therapy.

RESULTS IN PYODERMAS

The clinical results obtained in the 57 skin infections are shown in table II. Cases were graded as for acne. Adding the 21 pyodermas reported a year ago, we have a total of 78, with excellent clinical results observed in 87 per cent and good results in another 12 per cent.

* The trade name of Eli Lilly & Co. for erythromycin propionate is Ilosone.

† The trade name of The Upjohn Co. for tetracycline is Panmycin.

With only a few exceptions therefore skin infections caused by coagulase-positive *Staph. aureus* or beta-hemolytic *Streptococcus* respond to therapy with triacetyloleandomycin. In our series this held true whether or not the *Staphylococcus* was resistant to one or more antibiotics.

RESULTS IN MISCELLANEOUS INFECTIONS

A 50 year old white woman developed pneumonia as a complication of acute lupus erythematosus. She received 1 Gm. triacetyloleandomycin daily for one week with an excellent clinical response and no pulmonary relapse.

A good clinical result was recorded in a 12 year old white girl diagnosed as having a streptococcal throat infection and treated with 1 Gm. daily for seven days. A 45 year old white man with purulent bronchitis also received 1 Gm. daily for seven days with a good response.

SIDE EFFECTS

During the preparation of this paper, we learned of a report by Welsh and Ede, "Generalized Eruption from Triacetyloleandomycin."⁹ Dosage used by these authors was 250 mg. four times daily for 12 days.

In their series of 238 acne cases, Frank and Stritzler³ were obliged to discontinue therapy in 3 patients who developed a morbilliform eruption.

We have continued to be impressed by the freedom from adverse reactions of any sort. Prolonged therapy is feasible as evidenced by the use of the antibiotic in low dosage for 12 months in 70 acne patients. The patients with scleredema and cystic acne with nephritis received 1 Gm. of the drug daily for one month, and then 0.5 Gm. daily for another month. We never had to stop therapy in a single patient because of toxic reactions. On two occasions acne patients developed other pyodermic infections; merely increasing temporarily the dosage of triacetyloleandomycin served to control the pyoderma. We have found erythromycin propionate effective in staphylococcal infections, but untoward reactions forced its discontinuance in 2 patients.

Furthermore, there has been no development in vivo of bacterial resistance to triacetyloleandomycin that we could recognize.

DISCUSSION

Most of the 40 pustular acne cases reported a year ago were followed in our clinic. Patients who relapsed and were re-treated with triacetyloleandomycin showed the same beneficial results as with the original course of therapy. On re-treatment, none showed any signs of drug intolerance or any evidence of bacterial resistance.

In 1909 Fleming¹⁰ expressed his belief that the acne bacillus and *Staph. albus* were the etiological agents in acne. Recent studies by our group¹¹ strongly suggest that sensitivity to *C. acnes* plays a major role in cystic acne. Certainly patients with cystic lesions reacted more violently to the skin test with *C. acnes* vaccine. Also, hyposensitization with the acne bacillus-*Staph. albus* vaccine reduced this skin sensitivity to one half its original degree. Furthermore, hyposensitization served to

influence the type of lesion when patients relapsed—the lesions were smaller, and they showed very little induration and were noticeably less cystic.

We do not pretend to cure acne permanently, nor have we been able to control it with vaccine alone. Our preliminary observations indicate the need for studies with acne bacillus vaccine, *Staph. albus* vaccine or toxoid, a combination of both, and a placebo vaccine to delineate the respective roles of the etiological agents. At this early stage our impressions are these: Triacetyloleandomycin clears pustular acne but lesions recur when the antibiotic is discontinued. Vaccine desensitization markedly influences relapses in cystic acne, reducing lesion size, induration, and scarring.

SUMMARY

1. Triacetyloleandomycin and vaccine hyposensitization are useful and logical therapeutic procedures in the management of pustular and cystic acne.

2. Triacetyloleandomycin is effective in the treatment of skin infections caused by staphylococci or streptococci.

3. The use of triacetyloleandomycin in 178 patients has been attended by no adverse reactions or detectable bacterial resistance.

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The Role of Triacetyloleandomycin in the Treatment of Pustular and Cystic Acne: A Double Blind Study

WILLIAM J. MORGINSON AND Y. D. ESKELSON

*Dermatology Division, Department of Medicine, University of Utah
Medical School, Salt Lake City, Utah*

Most of us had used medium spectrum antibiotics in the treatment of acne, but these had been in short supply so that the dosage was less than desirable or the course of therapy too short. We welcomed a rather unlimited supply of the medium spectrum antibiotic with which to work.

Triacetyloleandomycin* and oleandomycin were distributed to each investigator in equal amounts but were identified only by number. The capsules were identical in color and shape. The dosage used was 250 mg. of either drug four times daily. Cases not followed for more than two weeks were not included. All types of acne were included in the series.

All patients had cultures obtained from their lesions. The bacteria were identified and cultural habits noted. Sensitivity tests were conducted on the bacteria in each case, and the degree of sensitivity was noted (table I). This work was done under contract by a registered laboratory.

Coagulase-positive *Staphylococcus aureus* was cultured from all the patients, and beta-hemolytic streptococci were also obtained from 20 per cent of the patients. The bacteria were equally sensitive in vitro to triacetyloleandomycin and oleandomycin.† More than 80 per cent showed 4+ inhibition (1 cm. in diameter of inhibition zone), 10 per cent showed 3+ inhibition (0.75 cm. in diameter of inhibition zone), 6 per cent showed 2+ inhibition (0.5 cm. in diameter of inhibition zone), less than 3 per cent showed no inhibition.

Patients were not questioned in regard to previous treatment or lack thereof. In most instances adjunctive therapy was continued, believing that familiarity with results of "standard therapy" would allow us to judge any change effected by the addition of the antibiotic to the therapeutic regimen.

One hundred and forty-two patients were placed on these drugs from four to eight weeks. Eighty-one per cent of the patients on triacetyloleandomycin had excellent or good therapeutic results. Sixty-four per cent of the patients on oleandomycin had excellent or good therapeutic results (table II). "Excellent" means a definite regression and control of the acne occurred. "Good" means that definite improvement occurred, but that the acne partially persisted. Four per cent of the total number of patients on both drugs had side effects, such as stomach ache or diarrhea. The drug was stopped and recovery was prompt.

* The trade name of J. B. Roerig & Co. Division, Chas. Pfizer & Co., for triacetyloleandomycin is Tao.

† Because of the relative insolubility of triacetyloleandomycin, oleandomycin is used in disc sensitivity testing for both compounds.

TABLE I

*Triacetyloleandomycin and Oleandomycin Sensitivity Studies:
Cultures Obtained from Pustules of 188 Acne Patients**

Organisms	Degrees of sensitivity	Diameter of inhibition zone	Number of patients
<i>Staph. aureus</i>	4 plus	1 cm.	127
	3 plus	0.75 cm.	18
	2 plus	0.5 cm.	13
	1 plus	0.25 cm.	0
	No inhibition	—	2
Total			160
<i>Staph. aureus</i> and beta-hemolytic <i>Streptococcus</i>	4 plus	1 cm.	23
	3 plus	0.75 cm.	2
	2 plus	0.5 cm.	2
	1 plus	0.25 cm.	0
Total			27
<i>Staph. aureus</i> and <i>Staph. albus</i>	4 plus	1 cm.	1

* Only 142 of these patients are included in the final study.

Many of the patients with the most acute types of acne had beta-hemolytic streptococci along with *Staph. aureus* in their cultures. These patients responded most quickly to antibiotic therapy and did so in two to four weeks. The improvement obtained at this time was about maximal for the drug. If therapy is stopped at this point, relapse will occur. The drug must either be continued or more reasonably adjunctive therapy must be relied upon to maintain or further the gains afforded by the antibiotic.

When the acne is of primarily comedone or comedo-papular type, broad-spectrum antibiotic therapy is of no help. If the acne is primarily cystic, the antibiotic will be of little help in clearing the cystic lesions. In other words, in the cystic cases it is still necessary to obliterate the cavities in order to effect a cure.

TABLE II

*Comparative Clinical Results with 2 Antibiotics in
Pustular and Cystic Acne*

	Triacetyloleandomycin		Oleandomycin	
	No.	%	No.	%
Excellent	28	31	11	21
Good	44	50	23	43
Negative	17	19	19	36
Total	89		53	

CONCLUSIONS

1. Triacetyloleandomycin is an important addition to the therapeutic regimen of pustular acne.
2. The more acute cases receive the quickest and most gratifying help.
3. Many of the more acute cases have beta-hemolytic streptococci in association with *Staph. aureus*.
4. Older therapeutic measures must be relied upon to maintain or further the improvement obtained from antibiotic therapy.
5. The more purely comedone type or more purely cystic type acne receive the least help from antibiotic therapy.

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Oral Demethylchlortetracycline in the Treatment of Pustular Dermatoses

NORMAN B. KANOF AND SAUL BLAU

New York, N. Y.

Systemic antibiotic therapy is used by dermatologists to combat primary infectious processes involving the skin and secondary pyoderma complicating various dermatoses and to control the pyogenic component of pustular and cystic acne vulgaris.

Demethylchlortetracycline,* a new antibiotic compound produced by a mutant strain of *Streptomyces aureofaciens* Duggar, is quickly and efficiently absorbed from the gastrointestinal tract, achieves immediate (i.e., one to three hours) serum concentrations equal to, or better than, those obtained with chlortetracycline, oxytetracycline, and tetracycline, and is as effective as or better than these compounds against assay organisms.^{1,2} Moreover, the serum antibiotic activity of demethylchlortetracycline declines more slowly than that of the other compounds and is still demonstrable 120 hours after oral administration, whereas the serum antibiotic activity of the other tetracyclines is no longer demonstrable 72 hours after ingestion. The prolonged activity in the serum of demethylchlortetracycline is probably due to its relatively slow renal clearance (43 per cent as rapidly as tetracycline³), but may also be due in part to its increased resistance to chemical degradation.⁴ These facts, together with the demonstration in animals and in human subjects that demethylchlortetracycline has a low order of toxicity, indicated its use orally in the treatment of pustular dermatoses.

METHOD AND RESULTS

Sixty-seven patients from dermatological practice were treated with demethylchlortetracycline orally. Forty-two were women and 25 were men. Their ages ranged from 9 to 64 years.

Each patient was supplied with capsules containing 150 mg. of demethylchlortetracycline. The usual initial dosage was 1 capsule four times daily (after meals and at bedtime). Each patient was observed regularly during the treatment period, which ranged from 4 to 16 weeks. At each visit, the patient's progress was recorded, the dosage of the medication adjusted according to the clinical response, and adverse reactions from therapy were noted.

The diagnoses of the patients included in this study and their responses to therapy are shown in table I.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin. The drug was supplied for this study by this firm.

TABLE I
Diagnoses and Responses to Therapy

Diagnosis	No. of patients	Response to therapy		
		Excellent	Good	Poor
Impetigo	2	2	—	—
Folliculitis barbae	2	1	1	—
Furunculosis	9	6	—	3
Hydradenitis suppurativa	1	—	1	—
Pustular rosacea	3	3	—	—
Miscellaneous dermatoses				
with secondary pyodermatization	5	4	1	—
Pustular acne vulgaris	45	26	12	7
Total	67	42	15	10

DISCUSSION

Twenty-two patients in this series had diagnoses of impetigo, folliculitis barbae, furunculosis, hydradenitis suppurativa, pustular rosacea, and pyoderma complicating other dermatoses. Nineteen patients (86 per cent) responded well. Those with impetigo, furunculosis, and secondary pyoderma (16 patients) showed a marked improvement in 48 to 72 hours, were clinically cured in seven days, and remained clear after treatment was stopped. In the 3 patients with furunculosis who failed to respond, bacterial studies showed the causative organism to be *Staphylococcus aureus*, phage type 80/81, very resistant to tetracycline. Those with folliculitis barbae, hydradenitis suppurativa, and pustular rosacea (6 patients) were better in one week and continued to improve during a six week period of therapy. Four of these patients remained free of pustular lesions when the drug was stopped, but the patient with hydradenitis and 1 of the patients with folliculitis barbae showed renewed pustular activity after cessation of therapy.

Forty-five patients had pustular acne vulgaris. Most of these patients were treated for 8 to 16 weeks, and the dosage of demethylchlortetracycline was reduced to 300 or 150 mg. daily after an initial period of one or two weeks at 600 mg. daily. Thirty-eight (84 per cent) showed "excellent" or "good" improvement. Fifty per cent of these showed some renewed pustular activity when demethylchlortetracycline was discontinued.

Two patients developed nausea and 2 diarrhea while on demethylchlortetracycline therapy. These side effects disappeared completely when the drug was discontinued. No pruritus ani or ulcerations about the mucous orifices, no urticarial or other drug eruptions occurred in this series of patients. None of the patients showed hematuria or albuminuria during therapy, and there was no instance of secondary monilial infection.

Several instances of photosensitivity to demethylchlortetracycline in the form of enhanced sunburn response to sun exposure have been reported to the manufacturer. We did not see any reactions of this type in our study.

SUMMARY AND CONCLUSIONS

Sixty-seven patients with pustular dermatoses were treated with demethylchlortetracycline by mouth and observed for periods of 4 to 16 weeks.

The initial dosage of demethylchlortetracycline was one 150 mg. capsule four times daily. When treatment was continued longer than two weeks (i.e., patients with pustular acne), the dosage of the drug was reduced to 1 capsule twice or once a day.

"Excellent" or "good" results were obtained in 57 patients (85 per cent). Those with impetigo, furunculosis, and secondary pyoderma were quickly and completely cured. Fifty per cent of the patients with pustular acne developed renewed activity when the antibiotic was stopped.

Nausea or diarrhea, which occurred in 4 patients, was completely and quickly reversible.

Demethylchlortetracycline is an excellent antibiotic for oral use in pustular dermatoses. The prolonged serum antibiotic activity attainable with a low dosage schedule of this medication is particularly useful in the extended treatment of pustular acne vulgaris.

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Observations on Treatment of Fungus Infections of Animals with Griseofulvin

G. A. GRECO, E. L. MOSS, JR., AND E. J. FOLEY

Biological Research Laboratories, Schering Corp., Bloomfield, N. J.

The efficacy of griseofulvin* in the treatment of dermatomycoses of animals and man is well established.¹⁻⁶ However, there is a paucity of published information on tissue distribution and excretion or on the efficacy of dosage schedules or treatment by routes of administration other than oral. The present experiments were undertaken to obtain information on several of these points.

MATERIALS AND METHODS

Dogs and guinea pigs were maintained individually in stainless steel metabolic cages for the collection of urine in excretion studies. They were dosed orally according to various schedules either with aqueous suspensions or with griseofulvin powder in soft gelatin capsules. Assays for antibiotic activity of feces or urine were made by diluting autoclaved samples (15 lb., 10 minutes) in Sabouraud broth. Moist feces were washed with petroleum ether and slurried with 5 volumes of methanol. Suitable dilution series were inoculated with agar discs seeded with freshly prepared suspensions from 10 day old agar slants of *Trichophyton rubrum*. Growth or inhibition was recorded after incubation for two weeks at 28 C. The particular assay strain was regularly inhibited in Sabouraud broth containing 1 µg./ml. but grew in a concentration of 0.5 µg./ml. The seeded agar plugs⁷ were prepared by inoculating 0.5 ml. of a saline suspension of spores with 9.5 ml. of melted and cooled (45 C.) Sabouraud agar. After the agar had hardened, 1 cm. plugs were cut out with a sterile cork borer and transferred to the tubes containing various amounts of antibiotic. Suspensions for injection were prepared by grinding with sterile saline or sesame oil in glass tissue grinders. Suspensions in corn oil were prepared similarly for oral administration. All doses in guinea pigs were given in a volume of 0.5 ml. For therapeutic experiments, white guinea pigs weighing 250 to 350 Gm. were caged in groups of 5. Skin infections were induced on abraded areas of the flank after clipping the hair. The method used was similar to that described by Molinas⁸ and subsequently employed by Gentles,¹ Martin,⁹ and Swartz et al.¹⁰ Lesions were observed and scored according to the severity of infection, and cultures were made from scales or vesicles at two day intervals over a 21 day observation period. The culture of *Trichophyton mentagrophytes* used for infecting guinea pigs was supplied by Dr. E. R. L. Gaughran. Additional details are given in the tables.

EXPERIMENTAL STUDIES

The pattern of urinary excretion in dogs after oral feeding at various dosage levels

* The trade name of Schering Corp. for griseofulvin is Fulvicin.

TABLE I

Antifungal Activity of Urine of Dogs Repeatedly Fed Griseofulvin

Dog no.	Day of experiment*	Daily dosage, Gm./Kg.	Total daily dosage, Gm.	Urine volume, 24 hr., ml.	Estimated antifungal activity, $\mu\text{g.}/\text{ml.}$	Total active drug excreted daily, mg.
H-56	1	2.0	19.78	21	4	0.084
	2	2.0	19.78	110	16	1.760
	3	2.0	19.78	132	8	1.056
	4	2.0	19.78	130	8	1.040
	5	2.0	19.78	155	8	1.240
EF-73	1	0.5	5.6	200	<4	—
	2	0.5	5.6	121	4	0.484
	3	0.5	5.6	176	4	0.704
	4	0.5	5.6	170	8	1.360
	5	0.5	5.6	138	16	2.208
EG-67	1	0.2	2.36	248	<4	—
	2	0.2	2.36	128	4	0.512
	3	0.2	2.36	114	4	0.456
	4	0.2	2.36	120	4	0.480
	5	0.2	2.36	155	8	1.240
EG-21	1	0.1	0.784	86	<4	—
	2	0.1	0.784	90	<4	—
	3	0.1	0.784	91	<4	—
	4	0.1	0.784	100	4	0.400
	5	0.1	0.784	104	4	0.416
EF-41	1	0	0	272	0	0
	2	0	0	350	0	0
	3	0	0	250	0	0
	4	0	0	240	0	0
	5	0	0	264	0	0

* Day 1 indicates the 24 hour period after the first dose of antibiotic.

was studied over a period of three weeks. It is seen in table I that only a fraction of the dose ingested was excreted in the urine. Little or none of the antibiotic was detected in the urine within the 24 hour period after the first administration of the drug and there was little evidence of cumulative excretion. Antibiotic was not detected in dog EG-21, which received 0.1 Gm./Kg., until the fourth day after treatment was begun. However, slight differences in total excretion are seen depending on the size of the dose administered. These same relationships are shown in table II, which demonstrates excretion patterns on the same dogs during the third week of feeding. Little difference in the total daily excretion was found between the dogs that received 0.5, 0.2, and 0.1 Gm./Kg./day, and it should be noted that excretion continued during the two days on which no drug was administered. Table III shows urinary excretion of griseofulvin over a period of seven days after three daily doses of 1 Gm./Kg. (days 1, 2, and 3). The antibiotic was excreted slowly, and detectable levels were observed for seven days after the last dose was given.

Similar experiments in which griseofulvin was given orally to guinea pigs are shown in table IV. These animals also excreted in the urine only a small fraction of the dose ingested. In experiments not shown in the tables, very low levels of 2 to 3 $\mu\text{g.}/\text{ml.}$ were detected in the blood of guinea pigs two to four hours after 50 Gm./

Kg. doses were administered orally. Small quantities of the drug were also recovered in the feces of these animals, approximately 0.5 per cent being recovered in samples collected over a 24 hour period after a single oral dose. None was recovered in the 24 to 48 hour period.

Results of treatment of experimentally induced *T. mentagrophytes* infections in guinea pigs are shown in table V. When treatment with oral aqueous suspensions was begun on the day that infection was induced, infection was prevented when 15 mg./Kg. doses of antibiotic were administered for 5, 10, or 15 days. Doses of 3 or 0.3 mg./Kg. were ineffective even when given for 15 days, although the severity of the infection was mitigated as compared with the untreated controls.

When aqueous suspensions were injected subcutaneously for 9 or 10 days, the

TABLE II
Antifungal Activity of Urine of Dogs Repeatedly Fed Griseofulvin

Dog no.	Day of experiment*	Daily dosage, Gm./Kg.	Total daily dosage, Gm.	Urine volume, 24 hr., ml.	Estimated antifungal activity, μ g./ml.	Total active drug excreted daily, mg.
H-56	1	2.0	20.5	142	8	1.136
	2	2.0	20.5	187	16	2.992
	3	2.0	20.5	126	8	1.008
	4	0	0	148	16	2.368
	5	0	0	184	8	1.472
	6	2.0	20.5	148	4	0.592
	7	2.0	20.5	216	16	3.456
EF-73	1	0.5	6.0	106	8	0.848
	2	0.5	6.0	100	8	0.800
	3	0.5	6.0	94	4	0.376
	4	0	0	120	4	0.480
	5	0	0	99	4	0.396
	6	0.5	6.0	160	4	0.640
	7	0.5	6.0	118	8	0.944
EG-67	1	0.2	2.35	119	8	0.952
	2	0.2	2.35	146	4	0.584
	3	0.2	2.35	158	4	0.632
	4	0	0	130	4	0.520
	5	0	0	285	4	1.140
	6	0.2	2.35	188	4	0.752
	7	0.2	2.35	151	8	1.208
EG-21	1	0.1	0.8	112	4	0.448
	2	0.1	0.8	94	4	0.376
	3	0.1	0.8	144	4	0.576
	4	0	0	217	4	0.868
	5	0	0	108	2	0.216
	6	0.1	0.8	140	4	0.560
	7	0.1	0.8	114	4	0.456
EF-41	1	0	0	490	0	0
	2	0	0	482	0	0
	3	0	0	414	0	0
	4	0	0	427	0	0
	5	0	0	345	0	0
	6	0	0	360	0	0
	7	0	0	378	0	0

* Dogs had been dosed continuously for two weeks prior to this period of collection of urine. Daily dosage continued during this period as indicated.

TABLE III
Antifungal Activity of Urine of Dogs Fed Griseofulvin

Dog no.	Day of experiment	Daily dosage, Gm./Kg.	Total daily dosage, Gm.	Urine volume, 24 hr., ml.	Estimated antifungal activity, μ g./ml.	Total active drug excreted daily, mg.
EI-45	1	1.0	7.6	220	8	1.76
	2	1.0	7.6	176	8	1.40
	3	1.0	7.6	280	8	2.24
	4	0	0	120	8	0.96
	5	0	0	100	4	0.40
	6	0	0	192	2	0.38
	7	0	0	150	2	0.30
	8	0	0	176	2	0.35
	9	0	0	212	2	0.42
	10	0	0	132	2	0.26
V-50	1	1.0	12.1	316	16	5.05
	2	1.0	12.1	284	64	18.17
	3	1.0	12.1	252	64	16.12
	4	0	0	172	32	5.50
	5	0	0	220	8	1.76
	6	0	0	270	8	2.16
	7	0	0	140	8	1.12
	8	0	0	176	4	0.70
	9	0	0	222	4	0.44
	10	0	0	190	2	0.38

15 mg./Kg. dose was without effect, 30 mg./Kg. modified the infection, and 60 mg./Kg. slowly effected a cure. Thirty mg./Kg. given by intraperitoneal injection for 10 days also resulted in a cure of infection by the twelfth day. Oil suspensions appeared to be more effective than aqueous suspensions when injected subcutaneously. Whereas the latter failed to cure when given at the 30 mg./Kg. level for 10 days, 30 mg./Kg. administered in oil completely prevented the infection, and 15 mg./Kg. was curative.

When treatment was withheld for 48 hours from the time infection was induced, higher dosages of aqueous suspensions given by the oral route were required to influence the infections. Oil suspensions given orally appeared to be more effective than aqueous suspensions at comparable dosage. When the oil suspension was injected subcutaneously, repeated doses of 40 mg./Kg. were required to cure. When treatment was delayed for 10 days, at which time extensive severe lesions existed, a series of 30 mg./Kg. doses of aqueous suspension administered for eight days effected cure of the infection.

DISCUSSION

The data in tables I, II, III, and IV show that only a small fraction of the griseofulvin ingested by dogs or guinea pigs is excreted in the urine. Similar observations have been reported in human beings.¹¹ Also, griseofulvin has been found in the hair of guinea pigs¹² when hot methanol was used for extraction. These results suggest that griseofulvin is absorbed readily and exists in a variety of tissues but is excreted slowly.

It is of interest that daily doses of 60 mg./Kg. were effective in curing *Trichophyton verrucosum* infection in calves² and *Microsporon canis* infections in cats¹³ and that doses of 30 to 40 mg./Kg. over a period of two to three weeks were effective in *M. canis* and *T. mentagrophytes* infections in dogs.¹⁴ In induced *T. mentagrophytes* infections in guinea pigs, oral doses of 60 mg./Kg. were effective regularly,^{1,10} and table V demonstrates that even smaller doses were sufficient to prevent or cure infections when treatment was started on the day infection was induced.

It appears that the response of infections of the abraded skin of guinea pigs depends on the dosage administered and that a higher dosage is required to cure infections when treatment is delayed after establishment of the lesion. When treatment was begun on the day of infection, 15 mg./Kg. doses of aqueous suspensions given orally for five days prevented infection. When treatment was delayed for two

TABLE IV

*Recovery of Griseofulvin in Urine of Guinea Pigs after Oral Administration**

Day of experiment	Guinea pig no.	Daily dosage, mg.	Urine		
			Volume, 24 hr., ml.	Estimated antibiotic, µg./ml.	Total antibiotic excreted daily, mg.
1	1	5	22	6	0.132
	2	5	78	8	0.624
	3	5	22	8	0.176
	4	5	48	8	0.384
	5	5	50	4	0.200
	6	5	50	8	0.400
2	1	5	25	8	0.200
	2	5	68	6	0.408
	3	5	48	8	0.384
	4	5	62	8	0.496
	5	5	55	8	0.440
	6	5	75	8	0.600
3	1	5	42	8	0.336
	2	5	62	6	0.372
	3	5	42	8	0.336
	4	5	32	8	0.256
	5	5	32	8	0.256
	6	5	48	8	0.384
4	1	0	20	6	0.120
	2	0	75	10	0.750
	3	0	38	10	0.380
	4	0	38	10	0.380
	5	0	15	10	0.150
	6	0	25	6	0.150
5	1	0	48	0	—
	2	0	75	6	0.450
	3	0	45	0	—
	4	0	75	0	—
	5	0	85	0	—
	6	0	58	6	0.348

* Guinea pigs, 250 Gm. Dose, 20 mg./Kg. Animals dosed 9:30 a.m. with aqueous suspension. Collections of 24 hour urine diluted in Sabouraud broth and autoclaved before inoculating with *T. rubrum*.

TABLE V

Influence of Dosage, Vehicle, and Route of Administration of Griseofulvin on Infections of Guinea Pigs

Experimental procedure, treatment begun	Duration of treatment, days	Dosage,* mg./Kg.	Route of administration	Vehicle, suspension	Result
On day of infection	5	15	Oral	Aqueous	Infection prevented†
	5	3	Oral	Aqueous	Mild infection
	5	0.3	Oral	Aqueous	Mild infection
	15	15	Oral	Aqueous	Infection prevented
	15	3	Oral	Aqueous	Mild infection
	15	0.3	Oral	Aqueous	Mild infection
	9	15	S.c. inj.	Aqueous	Severe infection
	9	3	S.c. inj.	Aqueous	Severe infection
	10	15	Oral	Aqueous	Infection prevented
	10	30	S.c. inj.	Aqueous	Mild infection
	10	60	S.c. inj.	Aqueous	Mild infection, cured by day 12
	10	30	I.p. inj.	Aqueous	Mild infection, cured by day 12
	10	30	S.c. inj.	Oil	Infection prevented
	10	15	S.c. inj.	Oil	Mild infection, cured by day 15
48 hr. after infection	6	20	Oral	Aqueous	Mild infection, persisted
	6	10	Oral	Aqueous	Severe infection
	6	5	Oral	Aqueous	Severe infection
	6	20	Oral	Oil	Mild infection, cured by day 12
	6	10	Oral	Oil	Mild infection, persisted
	6	5	Oral	Oil	Mild infection, persisted
	6	40	S.c. inj.	Oil	Mild infection, cured by day 8
	6	20	S.c. inj.	Oil	Mild infection, persisted
	6	10	S.c. inj.	Oil	Mild infection, persisted
	6	5	S.c. inj.	Oil	Severe infection
10 days after infection	8	30	Oral	Aqueous	Infection, cured by day 10

S.c. inj. = Subcutaneous injection; I.p. inj. = intraperitoneal injection.

* Dose administered in 0.5 ml. of vehicle.

† Cure or prevention of infection determined by successive negative cultures.

days before treatment was initiated, 20 mg./Kg. administered for six days failed to cure the infection.

There is some indication that griseofulvin is more efficacious when administered in an oil suspension than in an aqueous suspension. When 30 mg./Kg. doses were given for 10 days beginning on the day of infection, those guinea pigs receiving the oil suspension were cured. Similarly, when 20 mg./Kg. doses were given orally for six days beginning two days after infection, the oil suspension was effective whereas the aqueous suspension was not. Doses of 10 and 5 mg./Kg. in oil under these conditions, although not curative, gave a result superior to that obtained with aqueous injection. The 40 mg./Kg. dose was also effective when administered by subcutaneous injection for six days. Thus, griseofulvin is effective when given subcutaneously as well as orally, and it appears that the vehicle plays an important role in the result.

SUMMARY

Experiments are described that show that only a small fraction (less than 1 per

cent) of the dose of griseofulvin ingested by dogs is excreted in the urine. A low order of excretion also was observed in guinea pigs. On the basis of this and other data, it is presumed that the antibiotic is readily absorbed but is excreted slowly.

Response to experimental infections in guinea pigs depends on the dosage given, the route of administration, and the vehicle employed.

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Studies on the Mode of Action of Griseofulvin

E. J. FOLEY AND G. A. GRECO

Biological Research Laboratories, Schering Corp., Bloomfield, N. J.

After the demonstration that griseofulvin* when given orally is effective in the treatment of guinea pigs¹ and calves,² it was shown by Williams et al.,³ Blank and Roth,⁴ Riehl,⁵ and others to be effective in the treatment of dermatophytosis of man. Clinical results to date have been summarized by Sulzberger and Baer.⁶ Roth, Sallman, and Blank⁷ have shown that *Microsporum*, *Epidermophyton*, and *Trichophyton* are susceptible to griseofulvin in vitro. Concentrations of 0.1 to 0.2 µg./ml. retard growth of cultures as determined microscopically, and total inhibition of growth occurs in broth or agar containing 1 to 2 µg./ml. It is generally considered that griseofulvin is fungistatic rather than fungicidal.

The present experiments were undertaken to determine whether fungicidal action could be shown under conditions favoring rapid growth of fungi. They were prompted by certain clinical reports of marked improvement of lesions within a matter of days after treatment was begun and by observations on experimentally infected guinea pigs in which routine cultures from lesions quickly became negative after initiation of treatment.⁸

MATERIALS AND METHODS

All cultures were laboratory strains that had been transplanted in Sabouraud agar at six week intervals for at least two years. Under the conditions of standardized tests in Sabouraud broth, all were inhibited by 1 µg./ml. of griseofulvin and all grew in the presence of 0.5 µg./ml. For fungicidal tests, spore suspensions were prepared by washing 10 day old Sabouraud agar slants (incubated at 28 C.) with 4 ml. of sterile saline. The growth was gently rubbed with an inoculating loop to facilitate removal of spores. On the basis of microscopic counts, saline was added to the stock suspension in such an amount as to ensure a spore population of 10,000,000/ml. (\pm 10 per cent). Ten ml. of sterile Sabouraud broth (Difco) (in 150 \times 25 mm. Pyrex test tubes fitted with stainless steel closures), containing various amounts of griseofulvin, was inoculated with 0.1 ml. of the spore suspension. Subcultures were made at intervals by transferring 0.1 ml. volumes to unmedicated tubes of Sabouraud broth. These subcultures were incubated at 37 C. for 14 days, after which a final examination for growth of fungi was made. The various concentrations of griseofulvin were made by adding 0.1 ml. of concentrated ethanolic solutions to 10 ml. of broth. One tenth ml. of ethanol was also added to the control tubes, which did not contain griseofulvin. In order to rule out possible fungistatic effects due to a carry-over of griseofulvin, additional tubes of Sabouraud broth were inoculated with 0.1 ml. of the contents of each medicated tube in the series. Then these tubes were inoculated with approximately 10,000 spores of the various fungi and incubated along with the subcultures subsequently made in the test. In

* The trade name of Schering Corp. for griseofulvin is Fulvicin.

TABLE I
Fungicidal Action of Griseofulvin Against Various Dermatophytes

Test organism	Griseofulvin,* μg./ml. broth	Result of subcultures made at intervals,† hours						Fungistatic control
		0	4	8	24	36	48	
<i>T. rubrum</i> (S)	20	+	+	+	+	—	—	+
	10	+	+	+	+	—	—	+
	5	+	+	+	+	+	—	+
	2.5	+	+	+	+	+	—	+
	0.0	+	+	+	+	+	+	+
<i>T. mentagrophytes</i> (A-J)	20	+	+	+	+	—	—	+
	10	+	+	+	+	+	—	+
	5	+	+	+	+	+	—	+
	2.5	+	+	+	+	+	—	+
	0.0	+	+	+	+	+	+	+
<i>M. canis</i> (AT)	20	+	+	+	—	—	—	+
	10	+	+	+	—	—	—	+
	5	+	+	+	+	—	—	+
	2.5	+	+	+	+	+	+	+
	0.0	+	+	+	+	+	+	+
<i>M. canis</i> (QM)	20	+	+	—	—	—	—	+
	10	+	+	+	—	—	—	+
	5	+	+	+	+	—	—	+
	2.5	+	+	+	+	+	+	+
	0.0	+	+	+	+	+	+	+
<i>T. gypsum</i> (JS)	20	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+
	2.5	+	+	+	+	+	+	+
	0.0	+	+	+	+	+	+	+
<i>T. rubrum</i> (TT)	20	+	+	—	—	—	—	+
	10	+	+	—	—	—	—	+
	5	+	+	—	—	—	—	+
	2.5	+	+	+	+	+	—	+
	0.0	+	+	+	+	+	+	+

* Cultures exposed in griseofulvin broth at 37 C. during test period.

† Subcultures incubated at 37 C. Final readings made after 14 days.

no instance was a fungistatic effect observed. Additional details of the experiments are given in the tables.

RESULTS

It can be observed in table I that griseofulvin is fungicidal against all of the fungi tested under the conditions of these experiments and that there appears to be a marked difference in susceptibility among strains. Fungicidal action is more pronounced at the higher concentrations and as the time of exposure is increased. Similar results were obtained when the experiment was repeated with subcultures in Sabouraud agar instead of Sabouraud broth.

An experiment to determine whether the fungicidal effect is more pronounced under conditions that favor growth of fungi is shown in table II.

When fungi were exposed to griseofulvin at 4 C., all subcultures grew except the

TABLE II

Effect of Temperature on Fungicidal Action of Griseofulvin

Conditions of exposure of culture, <i>T. rubrum</i> (TT)		Results of subcultures made at intervals,* hours					Fungistatic control
Temperature, C.	Griseofulvin, $\mu\text{g./ml.}$	0	3	6	24	48	
Broth 4	20	+	+	+	+	—	+
	10	+	+	+	+	+	+
	5	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	0.0	+	+	+	+	+	+
12	20	+	+	+	—	—	+
	10	+	+	+	+	—	+
	5	+	+	+	+	—	+
	2.5	+	+	+	+	+	+
	0.0	+	+	+	+	+	+
37	20	+	+	—	—	—	+
	10	+	+	—	—	—	+
	5	+	+	—	—	—	+
	2.5	+	+	+	+	—	+
	0.0	+	+	+	+	+	+
Saline 4	20	+	+	+	+	+	+
12	20	+	+	+	+	+	+
37	20	+	+	+	+	+	+

* Subcultures incubated at 37 C. Final readings made after 14 days.

one made at 48 hours from the tube containing 20 $\mu\text{g./ml.}$; of those exposed at 12 C., all grew except those made at 24 and 48 hours from the tubes containing 20 $\mu\text{g./ml.}$ and those made at 48 hours from the tubes containing 10 and 5 $\mu\text{g./ml.}$ The killing effect was most pronounced in tubes held at 37 C. Subcultures made after six hours from tubes containing 20, 10, and 5 $\mu\text{g./ml.}$ failed to grow. In contrast to these results obtained in broth, all subcultures made from tubes containing 20 $\mu\text{g./ml.}$ of griseofulvin in saline were viable. These results suggest that griseofulvin exerts its effect more vigorously in fungi that are actively metabolizing, and if this is so, the action resembles that of penicillin.^{9,10}

In experiments by Roth et al.,⁷ in which pellets of growth of *Trichophyton rubrum* or *Trichophyton mentagrophytes* obtained by cultivation in broth on a gyratory platform were exposed to 25 $\mu\text{g./ml.}$ concentrations of griseofulvin and then washed and seeded on agar, no evidence of fungicidal effect was noted. We have conducted experiments under the same conditions with *T. rubrum* and *T. mentagrophytes* with similar results.

Differences in technique probably account for the divergent results of Roth et al.⁷ and those described here. If fungicidal effect depends on interaction of griseofulvin and actively metabolizing fungi, it would seem that the discrete spores and hyphae of roughly similar age that we used as our inocula would have a better chance of growing actively than would the varied elements that constitute the growth of circumscribed pellets, which develop progressively over a five day incubation period. It is conceivable that viable fungi in the center of the mass would grow at a

lesser rate than those at the periphery and thus be less likely to respond to the action of the antibiotic.

Whether the fungicidal effects observed in broth in the present experiments have implications in the control of fungus infections of the skin or hair after oral dosage of griseofulvin is open to speculation. On the basis of studies by Gentles et al¹¹ in which 1 Gm. of hair from guinea pigs fed for three weeks with griseofulvin yielded only 5 to 6 μ g., it would not be expected that an effective fungicidal concentration is achieved in infected structures. However, we know little of the rate of growth of fungi in hair or skin or of the mechanism of action of griseofulvin in natural infections. Presumably, during treatment the fungus encounters the antibiotic and its growth is greatly reduced, and being held in a matrix of antibiotic-containing cells or keratin, it is eventually removed by normal processes of skin erosion.

SUMMARY

Experiments are described in which griseofulvin is fungicidal against a mixed inoculum of spores and hyphae of *Trichophyton* or *Microsporum*. It is suggested that griseofulvin exerts its fungicidal effect under conditions that are conducive to active growth of the fungi.

ACKNOWLEDGMENT

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Metabolic Studies on Griseofulvin and Its Mechanism of Action

EARL G. McNALL

Department of Medicine, Division of Dermatology, University of California Medical Center, Los Angeles, Calif.

Griseofulvin is a spirane derivative that was originally isolated from *Penicillium griseofulvum* by Oxford et al in 1939.¹ Brian et al isolated a "curling factor" from cultures of *Penicillium janczewski*, which was later proved to be identical with griseofulvin.^{2,3} The chemical structure of this compound was established and its activity against a variety of fungi reported.⁴⁻⁸ Several workers have reported the efficacy of this compound in the systemic treatment of dermatophyte infections in man and experimental animals.⁹⁻¹¹ Blank and Roth were unable to demonstrate antibiotic activity from the serum of patients by a direct biological assay.¹² These workers, however, reported that more than 50 per cent of the orally administered drug could be demonstrated in the urine by the same biological assay.

This paper reports the blood levels of griseofulvin as measured by a direct bioassay and radioactive assays and in vitro studies that may provide evidence for the mechanism of action of this antibiotic.

METHODS

Microbiological Assay. The concentration of griseofulvin in body fluids may be determined by measuring the growth inhibition of a griseofulvin-sensitive organism. Griseofulvin-sensitive strains of *Microsporum canis* and *Microsporum fulvum* were grown in the assay media and transferred in the logarithmic phase of growth, centrifuged at approximately $1000 \times g$ for 10 to 15 minutes, washed with sterile water, and ground in a Ten Brock tissue grinder, and made up to 10 to 15 per cent suspension in growth medium. The growth media consisted of mycophil broth supplemented with 0.1 per cent yeast extract, 500 mg./ml. of cycloheximide, and 100 units/ml. of neomycin. At these concentrations no inhibition of growth of *M. canis* or *M. fulvum* was observed. Blood was collected from human beings by venipuncture and from rats and mice by cardiac puncture, allowed to clot for two hours at room temperature, and centrifuged at 1 to $2000 \times g$ for 30 minutes followed by heat inactivation of the clear unhemolized sera at 56 C. for 30 minutes, and stored at -20°C . until assayed for activity. The naturally occurring antifungal activity in sera is largely destroyed by the heat inactivation in the latter step. Sera so treated consistently stimulated the growth of the organism beyond that obtained with the medium alone. The total volume of sera containing griseofulvin from man or rat was adjusted to 1.0 ml. All dilutions were made in sera, and because of the relatively low concentration of the drug in such sera, usually duplicates of undiluted sera and dilutions of 1:1 and 1:2 were used. One ml. of the 10 to 15 per cent

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TABLE I
Inhibition of Growth of Griseofulvin

Griseofulvin, μg./flask	Growth volume of packed mycelia, ml.	
	<i>M. canis</i>	<i>M. fulvum</i>
10	0.6	.1
6	1.0	.2
2	4.3	0.4
0.4	5.5	1.4
0.2	6.5	2.0
0.1	7.0	2.8
0	8.0	3.3

suspension of inoculum was added to 1 ml. of the serum containing griseofulvin in 50 ml. Erlenmeyer flasks and incubated at 25 C. for four hours. Ten ml. of the assay medium was added to each flask and the incubation continued on a shaker at 25 C. under conditions that provide vigorous aeration for a period of 36 to 48 hours. A standard curve was prepared in duplicate covering a range of 1/10 to 10 mg./ml. in a final volume of 1 ml. of serum and treated as described. Each flask was further incubated for 48 hours at 25 C. with vigorous aeration. The contents of each flask was poured into a 15 ml. calibrated conical centrifuge tube and centrifuged at $1 \text{ to } 2000 \times g$. for 30 minutes. The packed mycelial volume of each tube was measured and the concentration of the drug was estimated from comparison with packed mycelial volumes of the tubes containing known quantities of griseofulvin.

Assays Utilizing Tritium-labeled Griseofulvin. One and one half Gm. of griseofulvin was exposed to an atmosphere of 3 curies of tritium gas at 27 C. and 0.39 atmosphere pressure for two weeks. The final activity of the randomly labeled compound after recrystallization was 0.19 mc./mg. No decrease of specific activity was observed after several recrystallizations. Swiss-Webster mice were injected intraperitoneally with tritiated griseofulvin equivalent to 20 mg./Kg. Blood was withdrawn from the heart at 1, 3, 8, and 24 hours and allowed to clot, after which 0.1 ml. of each serum was pipetted into 0.5 ml. distilled water. The blood was allowed to hemolyze for 30 minutes, after which it was extracted with 1 ml. of toluene by shaking intermittently for 10 minutes. The counting efficiency was regularly between 15 and 20 per cent. Accurately standardized tritium-labeled

TABLE II
Serum Griseofulvin Levels after a Single Oral Dose of 1 Gm.

Time, hours	Range, μg./ml.	Average griseofulvin level
1	Less than 0.1 mg.	
3	0.2-0.6	1.3
4	0.5-3.3	1.9
8	0.4-3.8	2.0
9	0.5-1.3	0.6
24	0.2-0.6	0.4

TABLE III

*Griseofulvin Concentration in Rat Serum as Measured with Biological Assay
after a Single Intraperitoneal Injection*

Time, hours	Serum griseofulvin, $\mu\text{g.}/\text{ml.}$
50 mg./Kg. Injected	
1/2	0.5
1	2.0
3	2.1
24	0.9
200 mg./Kg. Injected	
1/2	0.8
1	2.8
3	2.6
24	1.7

griseofulvin was added to the blood-water mixture and extracted by the same procedure. The recovery of radioactivity was 95 to 97 per cent of the added tritiated compound. One half ml. aliquot of toluene from each sample was removed and added to 4.5 ml. of scintillation solution. The scintillation solution was composed of diphenyloxazole, 3 Gm./liter, and 1,4-di (2-(5-phenyloxazolyl))-benzene, 20 mg./liter, in reagent grade toluene. Counting periods were sufficiently long to ensure good statistics, i.e., deviations of $\sim \pm 2$ per cent at 95 per cent confidence limits.

EXPERIMENTAL RESULTS

The standard curve of fungal growth as a function of griseofulvin concentration shows detectable growth inhibition by both organisms at 0.1 $\mu\text{g.}$ at the lowest level through 10 $\mu\text{g.}$ at the highest level. The slope is greatest and the assay data are most reproducible between 0.2 and 1 $\mu\text{g.}/\text{ml.}$ (table I). Serum griseofulvin levels of 6 adult male volunteers after a single oral dose of griseofulvin are shown in table II. The levels vary considerably from individual to individual; however, the maximum serum levels of griseofulvin were usually observed between four and eight hours. Rats injected intraperitoneally with griseofulvin exhibited a maximal level between one half and one hour. The concentration of this drug decreased to approximately one half the maximal level in 24 hours (table III). The serum levels of radioactive griseofulvin after a single intraperitoneal injection in mice produced a similar pattern to that observed in rats using the biological assay. Again, the

TABLE IV

*Tritiated Griseofulvin Concentration in Mouse Serum after a Single
Intraperitoneal Injection of 10 mg. Body Weight*

Time, hours	Concentration of griseofulvin, $\mu\text{g.}/\text{ml.}$
1/2	5.4
1	9.7
3	9.9
8	10.1
24	5.9

TABLE V
*Reversal of Griseofulvin-Induced Growth Inhibition
by Purines and Purine Derivatives*

Compound	Growth
Griseofulvin control	0
Adenylic acid	++
Guanylic acid	++
Adenine	+
Guanine	+
Adenylic acid plus guanylic acid	++++
Adenine plus guanine	++

All flasks contained 1 μ g. of griseofulvin in 10 ml. of brain-heart infusion broth and 0.5 ml. of a 20 per cent suspension of *M. canis*. Purines and purine derivatives were at a concentration of 10 μ g./flask. 0 = No growth. +, ++, +++ = Approximately 10, 25, and 50 or more per cent growth of control flasks lacking griseofulvin.

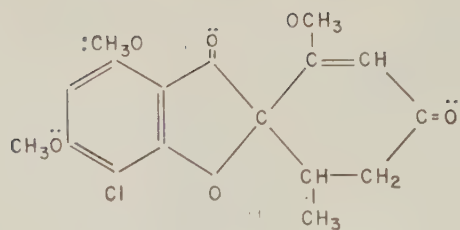
maximal levels were attained within the first one half to one and one half hour period and decreased to two thirds the maximal level at 24 hours (table IV).

Inhibition of growth of *M. canis* by griseofulvin was partially reversed by purines and purine derivatives to varying extents (table V).

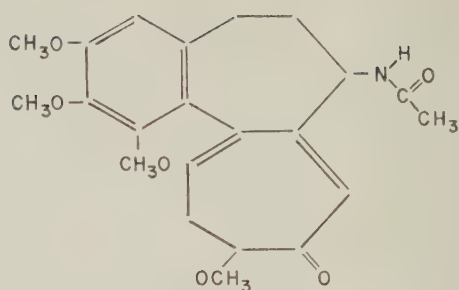
DISCUSSION

Griseofulvin may be measured by direct biological assay. It is important, however, to maintain a constant concentration of serum because of the marked growth stimulation imparted by serum in the growth media. Unless the serum concentration is kept constant, growth inhibition by griseofulvin may be masked by the stimulating effect of serum. Sera that are not heat inactivated produce effects ranging from strong inhibition to stimulation of the growth of the organism. Studies in our laboratory have shown that heat-labile antifungal activity is present in both animal and human sera.¹³ Without prior heat inactivation, it is possible to obtain anomalous results, which would result from a composite of stimulation and inhibition by the sera. The time course of griseofulvin serum concentration as measured by the biological assay in human beings after a single oral dose of 1 Gm. varies considerably and may be influenced by diet, intestinal mobility, and other parameters that could not be controlled in our study. Furthermore, this assay detects only biologically active forms of this drug. There is evidence that a portion of this antibiotic is reduced from the ketone level to the primary alcohol on the spirane ring.¹³ The latter compound lacks antifungal activity and could be detected only by the use of radioactive griseofulvin or by spectrophotometric or spectrofluorometric assays. The latter three types of assays detect both the biologically active and inactive derivatives of griseofulvin.

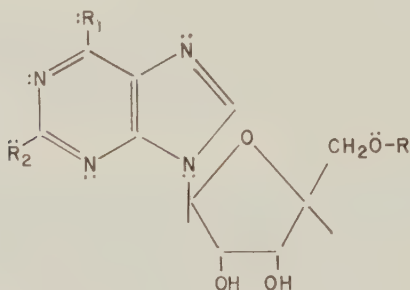
The partial reversal of growth inhibition by purines and purine derivatives supports the hypothesis that the antifungal activity of griseofulvin is due at least in part to its inhibition of nucleic acid synthesis, at steps either prior to or at the polymerization stage. When this antifungal antibiotic is injected intravenously at approximately 10 times (100 to 200 mg./Kg.) the therapeutic dosage, arrest of mitosis is observed in metaphase.¹⁴ Tissues most dramatically affected in the rat are bone marrow, mucosal



GRISEOFULVIN



COLCHICINE



PURINE RIBOSIDE

FIGURE 1.

epithelial cells, seminiferous tubules, and proliferating transplanted tumors.¹⁴ Nucleic acid turnover and the mitotic indexes of these tissues are among the highest in the mammalian body.

Colchicine, a chemically unrelated compound (fig. 1), induces similar effects; however, colchicine per se does not inhibit the growth of fungi that are known to be sensitive to griseofulvin, even at high concentrations.¹⁵ Griseofulvin does not exhibit the severe toxic manifestations in experimental animals that is seen with colchicine at levels that produce the same inhibition of mitosis.¹⁴ It is well known that colchicine inhibits mitosis by disorganizing spindle formation.¹⁶ The spindle apparatus, when affected by colchicine, loses its high degree of orientation and behaves like a gel.¹⁶ The result, especially in plant cells, is the formation of polyploid cells; reduplication of chromosomes occurs normally, but the latter are unable to migrate toward the poles of the spindle. If the cell survives and a nuclear membrane is formed, polyploid cells result. Paget and Walpole stated that griseofulvin appears to induce fewer polyploid cells than colchicine.¹⁴ Although there are certain similarities in the net action of griseofulvin and colchicine on mitosis, further studies should be made to determine whether the spindle apparatus is affected in a manner similar to that with colchicine.

The inhibition of mitosis at metaphase by griseofulvin may also be explainable in terms of direct inhibition of nucleic acid metabolism. The intramolecular distances between the methoxyl oxygens on the benzene ring and the ketone on the spirane ring are very similar to the distances from nitrogen or oxygen of the R1 and R2 of the purine ribosides and the primary alcohol group (fig. 1). It may be noted that an unshared pair of electrons is present on each of the previously

mentioned atoms. There are several other sites on the two molecules where a pair of electrons on oxygen and nitrogen present a picture of structural similarity. The first two rings of griseofulvin resemble in many structural ways the first two rings of the purine molecule. These structural similarities suggest strongly that griseofulvin may possibly act as competitive analogue for nucleic acid synthesis. If the interference of nucleic acid is due to inhibition of polymerization, indeed it might be expected that inhibition would occur as it does at metaphase.

Inhibition of growth by griseofulvin cannot be explained on the basis of interferences with the synthesis of chitin in the cell wall, as suggested by other workers,¹⁷ since a large variety of fungi that contain chitin are not inhibited and many that are inhibited do not contain appreciable quantities of this polymer.¹³ It is more appealing to consider the possibility of a generalized inhibition of nucleic acid metabolism, which would be expected to cause a decrease in the ability of the organism to synthesize protein and the more complex cellular constituents and polysaccharides that make up the walls of these organisms.

SUMMARY AND CONCLUSIONS

Griseofulvin may be assayed in biological fluids by direct microbiological assay. The concentrations of griseofulvin in man, rat, and mouse after various levels and routes of administration are reported. The inhibition of griseofulvin-sensitive fungi can be reversed partially by purines and purine derivatives. The implications of the latter findings are discussed in terms of inhibition of nucleic acid synthesis.

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Griseofulvin Therapy of Superficial Mycoses

MURRY M. ROBINSON

Washington, D. C.

Griseofulvin, which is highly selective in its effect on various dermatophytes, seems specific in its action on the *Trichophyton*, *Microsporum*, and *Epidermophyton* groups of the fungi.

Although seldom serious, the superficial mycoses have been quite troublesome. The incidence increases progressively as one goes from frigid to tropical climates. Although the mortality rate is quite low, the morbidity rate is extremely high.

The ubiquity of the superficial dermatophytes is well known. As far back as 1930, I was able to grow these dermatophytes from centrifuged specimens of water collected from the floors of gymnasium showers and locker rooms and from centrifuged specimens of washings of bath mats freshly laundered by commercial laundries. It has been estimated that the majority of shoe-wearing men have some degree of tinea pedis. How important a disease this has been in the past is demonstrated by the following quotation from Pillsbury et al:¹ "In 1942 and 1943, when the battle of the Atlantic was in the most precarious balance, and when the supply of food and ammunition in England was constantly bordering on the inadequate, foot baths for the prevention of athlete's foot were dutifully being shipped from America in large numbers."

Treatment until now has been limited to topical agents, many of them odorous, bothersome, and ineffective, with which it was attempted unsuccessfully to penetrate the keratotic layers and destroy the fungus from the outside in. Some extremely superficial mycoses of the glabrous skin would yield to persistent topical therapy, but infection of the finger- and toenails presented almost hopeless therapeutic problems. A new therapeutic agent that would be effective when administered systemically was badly needed; such an agent is griseofulvin.

CHEMISTRY

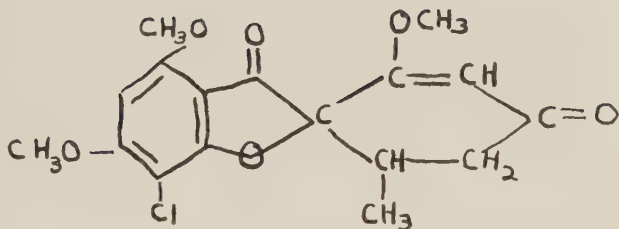
Griseofulvin is a colorless, slightly bitter, thermostable compound, which chemically is 7-chloro-2', 4, 6 trimethoxy-6'-methylspiro benzofuran-2 (3H), 1'-(2) cyclohexene-3, 4' dione.

The structural formula of griseofulvin is shown in figure 1. At pH 7 griseofulvin is slightly soluble in water, 0.001 per cent, methanol, 0.1 per cent, ethanol, 0.1 per cent, and N,N-dimethylformamide, 12 per cent. In solution at the same pH, it is thermostable, withstanding autoclaving at 250 F. for 30 minutes; at 100 F. it is stable in the dry form for at least 20 months.

SPECTRUM OF ACTIVITY

Griseofulvin inhibits the growth of the following fungi that commonly infect the hair, nails, and skin: *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton*

FIG. 1. Structural formula of griseofulvin.



mentagrophytes, *Trichophyton interdigitale*, *Trichophyton verrucosum*, *Trichophyton sulfureum*, *Trichophyton schoenleini*, *Microsporum audouini*, *Microsporum canis*, *Microsporum gypseum*, and *Epidermophyton floccosum*.

Griseofulvin is not effective against bacteria, histoplasmosis, candidiasis (moniliasis), actinomycosis, sporotrichosis, chromoblastomycosis, coccidioidomycosis, cryptococcosis (torulosis), North American blastomycosis, *Malassezia furfur*, or nocardiosis.

HISTORY AND CHARACTERISTICS OF GRISEOFULVIN

Griseofulvin is a fermentation product of three species of *Penicillium*: *Penicillium griseofulvum* Dierck X, *Penicillium janczewskii*, and *Penicillium patulum*. It was discovered by Oxford et al² in 1939 and its antifungal qualities were described by Brian and his co-workers.^{3,4} They noted that in low concentrations it suppressed growth, and produced excessive branching with considerable distortion of the hyphal elements of a number of fungal species. This effect on the hyphae was termed the "curling factor" by McGowan.⁵ Grove et al⁶ described the structure of griseofulvin as C₁₇H₁₉O₈Cl, which was substantiated by other investigators.^{7,8}

In the decade after the discovery of griseofulvin, some investigation continued on a purely scientific basis. It was found that other varieties of *Penicillium*, namely *Penicillium raistrickii*⁹ and *Penicillium nigricans*,¹⁰ were also capable of producing griseofulvin. No one attempted to use the drug clinically until it was found, in 1955, by workers at the Glaxo Laboratories, that griseofulvin in vitro inhibited the growth of many pathogenic dermatophytes. It took about two decades, however, after its discovery before Gentles,¹¹ after infecting guinea pigs with *M. canis* and *T. mentagrophytes*, demonstrated the in vivo effect of griseofulvin when given orally. He administered the drug in doses of 60 mg./Kg. and noted that in the treated animals only the tips of the hairs fluoresced under filtered ultraviolet light, and that on microscopic examination the dermatophyte could be found only in the portion of the hair shaft that fluoresced.

A beneficial effect was noticed within four days after treatment began. It was evident that no dermatophytes existed at the base of the hair shaft and that this clear part was sharply delineated from the infected hair tips. It was further evident that something had occurred to make that portion of the hair shaft that had grown while the animal was under treatment resistant to the invasion by the dermatophyte. The reason for the apparent effect on guinea pig hair was made clear when it was demonstrated that griseofulvin was absorbed from the gastrointestinal tract and deposited in the keratin of the hair.¹² Launder and O'Sullivan¹³ later demonstrated that the drug was effective in the treatment of induced *T. verrucosum* in cattle.

TABLE I

Diagnosis

	Tinea glabrosa	Tinea cruris	Tinea pedis	Onychomycosis
No. of cases	13	7	15	17

Treatment of human beings was first attempted by Williams and his group¹⁴ in 1958. They treated 9 patients infected with *T. rubrum* and *M. audouini*, with excellent results. Numerous studies have been done since then and all have given good results. Recent studies by many workers¹⁵⁻¹⁹ in the field attest to the amazing efficacy of griseofulvin when used properly. In tinea capitis it has made radiological epilation completely obsolete. Onychomycosis due to infection with *T. rubrum* is now curable for the first time.

The topical use of the drug was first tried by Pardo-Costello²⁰ in 1957. He reported some improvement except in tinea capitis but no cure in any of his 10 cases. In 1959 Martin²¹ reported that topical application for treating induced ringworm in guinea pigs gave good results, but not so good as when the drug was used orally.

DOSAGE SCHEDULE

The consensus of other workers, and my own experience, indicated that in adults 1 Gm./day in four divided doses is optimum. In children weighing up to 50 lb., 500 to 750 mg. in divided doses is used; for children above that weight the adult dosage is used. In some cases increased dosage may be necessary and seems to be well tolerated.

EVIDENCE OF TOXICITY IN EXPERIMENTAL STUDIES

Studies in animals indicate that massive dosages of griseofulvin can be administered orally without causing death. Mice survived oral doses of 50 mg./Kg.²²

Paget and Walpole²³ reported that the seminal epithelium in rats was severely damaged when large intraperitoneal doses (2 Gm./Kg.) were administered. When doses of 100 to 200 mg./Kg. were administered intravenously, there was an arrest

TABLE II

Infecting Organism

	<i>T. rubrum</i>	<i>T. mentagrophytes</i>	<i>T. schoenleini</i>	<i>E. floccosum</i>
Tinea glabrosa	8	2	1	2
Tinea cruris	3	2		2
Tinea pedis	10	5		1
Onycho- mycosis	13	3		1

of mitosis at the metaphase. These changes were reversible, with recovery occurring in most tissues within 24 hours after a single dose. Interference with spermatogenesis was still evident 10 days after intravenous administration of the drug. Further studies are being continued in order to discover possible toxicity. Blank¹⁶ determined that there was no change in the karyokinetic index.

METHODS AND MATERIALS

Fifty-two patients with various types of superficial mycoses form the basis for this experiment. The division according to diagnosis is shown in table I and the division according to organism is shown in table II.

No patient was put on griseofulvin therapy unless positive laboratory evidence indicated the infective agent to be a *Trichophyton*, a *Microsporum*, or an *Epidermophyton*. Prior to treatment a laboratory base line was established. The blood and urine were examined and pretreatment liver function was determined. A battery of liver function tests was employed, including icterus index, thymol turbidity, alkaline phosphatase, and serum transaminase.

These laboratory studies were repeated every two weeks while the patient was under therapy. The urine was checked at least once weekly, as were the patient's weight and blood pressure. Cultures were repeated at intervals during therapy.

Griseofulvin was administered to all patients in a dosage of 250 mg. four times daily. There were occasional accidental changes in therapy. One patient had to have an increased dosage.

Patients were considered cured when all evidence of the mycosis had disappeared and when potassium hydroxide preparations and cultures were negative.

RESULTS

Tinea Glabrosa. Subjective symptoms were relieved within two to six days regardless of etiology or duration. The minimum time for cure was six weeks; the maximum time has not been ascertained since 1 patient has been on griseofulvin for more than 14 weeks and signs of lesions are still present. This patient developed ringworm while on massive dosage of steroids for the treatment of pemphigus, and although the condition is markedly improved, there are still vestiges of the original lesions. The average period required for cure is 10 weeks.

Tinea Cruris. Subjective symptoms were relieved in two to four days. Complete laboratory and clinical cure occurs within 8 to 12 weeks.

Tinea Pedis. Subjective symptoms were relieved in three to six days. It took somewhat longer (an average of 16 to 24 weeks) to cure tinea pedis than tinea cruris and tinea glabrosa. This is probably due to the presence of yeasts and bacteria as complicating factors.

Onychomycosis. Evidence of improvement of this condition can be seen in the fingernails within two weeks and in the toenails in about three to four weeks. The area of clear nail zone is sharply demarcated from the infected portion. It is amazing to see that week after week this clear zone of healthy nail becomes wider and wider.

In several patients in whom the infection affected only the nails of one hand or

one foot, the distance from the proximal end of the infected area to the cuticle was measured with a pair of calipers. On the corresponding nail on the unaffected extremity, the same distance was measured off and a file mark made so that a permanent artefact was created that could be observed. The rate at which the file mark on the unaffected nail and the line delineating infection on the other side progressed distally was measured at biweekly intervals. In both cases growth was equal. In the fingernails the rate of growth was just over 1 mm. weekly, while on the other toenails it was approximately 0.5 mm. weekly. From these simple calculations, it was possible to estimate the time it would take to cure the average case of onychomycosis.

Therapeutic results in all cases were considered excellent. In 1 case, all of the nails cleared after seven months of therapy with the exception of the fourth toenails on each foot, which were unchanged and from which fungi could be recovered repeatedly. In 1 patient, there was an exacerbation in one nail which subsided when the dose was increased from 1 to 1.5 Gm. daily.

SIDE EFFECTS

One patient developed excessive fatigue and severe diarrhea, which made it necessary to discontinue treatment.

Three patients developed vague feelings of gastrointestinal uneasiness after the first three to seven days of therapy. These later disappeared although the drug was continued.

One patient complained of a feeling of "fullness" in the head, confusion, exhilaration, and insomnia. It was originally thought that this patient's symptoms were psychogenic because she was known to be hypertonic and easily influenced by suggestion. A different brand of griseofulvin was substituted, which in no way resembled the original tablet in color, size, or shape. It was then suggested to her that this was a different drug, which would not produce the untoward side effects. After two days the same symptoms reappeared.

Fifty per cent of all patients treated had a decrease in systolic blood pressure of between 10 and 15 per cent, without any subjective symptoms. Twelve patients developed a decrease in the leukocyte count, averaging about 1500 cells/cu. mm. Of these, 4 developed a relative lymphocytosis. Three additional patients whose leukocyte count was basically unchanged also developed a relative lymphocytosis. Five patients developed very mild albuminuria varying from 1 to 2 plus. Kidney function tests done on these patients revealed no abnormality; therefore, no explanation for this phenomenon can be given at this time.

One patient complained that she developed a tachycardia and a flush if she imbibed any alcohol. Because this may have been purely a subjective symptom of psychogenic origin, she was instructed to repeat the performance again. She again developed a tachycardia and flushing.

Three patients in the group developed severe urticaria as a result of either ingestion or injection of penicillin. None of these patients developed any untoward side effects from griseofulvin.

In 2 patients there was an elevation of the icteric index without any visible evidence of jaundice.

Summary of Side Effects. One patient was forced to discontinue treatment because of excessive fatigue and severe diarrhea. One patient stopped taking the drug because of exhilaration, insomnia, and confusion. Some slight gastrointestinal symptoms were seen in 3 patients. One patient had a recurrence or a reinfection six weeks after declared cured. One patient developed a separation of one thumb-nail while still under treatment.

All other patients responded excellently to griseofulvin.

SUMMARY AND COMMENT

Many side effects have appeared in patients in this series. In only 2 cases were side effects severe enough to discontinue treatment, but certainly all should be noted and investigated further.

Griseofulvin, which to date is so effective in the treatment of the epidermophytoses, will be of great benefit to patients suffering from these mycoses. I expect, however, that in spite of its efficacy, there will be numerous occasions on which it will be discredited. The adverse criticism can be expected because of its failure as a therapeutic agent when it is prescribed for dermatoses, which are not mycoses or are not produced by either trichophytos, microsporons, or epidermophytos.

I think I can safely predict that it will become one of the most abused of drugs. Many dermatoses are labeled "fungus infections," although their true character may be entirely different. Not every ringlike lesion is ringworm, and not all ringworm has a ringlike configuration. Also, fungus infection produced by organisms other than those of the *Trichophyton*, *Microsporum*, or *Epidermophyton* group cannot be expected to respond to griseofulvin therapy. Fungi also occur as innocent invaders. For example, it is usual for aspergilli to invade the external auditory canal that has been made boggy by immersion in a swimming pool or has become infected by *Pseudomonas aeruginosa* as a result of a basic seborrheic dermatitis. This condition has frequently been diagnosed as a fungus infection of the ears and treated with antimycotic agents, the most popular of which is Castellani's paint. It is not to be expected therefore that griseofulvin will be effective in this type of otitis.

Griseofulvin should never be administered unless there is positive laboratory evidence of an infection with a susceptible organism, except in the most unusual circumstances when potassium hydroxide preparation and culture are negative, but when the clinical manifestations are indisputable. Even in such cases the keenest judgment should be tempered by honest doubt.

The drug is too new to permit accurate predictions as to its effectiveness and toxicity. As time goes on, and as the use of griseofulvin becomes universal, as it surely will, many factors will be evident that are now obscured. If, however, its early promise is substantiated by time and trial, its introduction will prove of great value, and man will have been made just a little happier.

ADDENDUM

At the time this paper was sent to be published, certain facts were unknown. Since that time it has been revealed by Dr. Latapi of Mexico that the drug has some

therapeutic effect in nocardiosis and in sporotrichosis. Dr. Gonzales-Ochoa, also of Mexico, reports some effect on chromoblastomycosis. Some workers in as yet unpublished papers have reported side effects similar to the ones described in this paper.

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Laboratory and Clinical Observations in the Therapy of Dermatomycoses with Griseofulvin

STURE A. M. JOHNSON AND GEORGE H. CAMERON

Department of Dermatology, University of Wisconsin Medical School, Madison, Wis.

Griseofulvin* was originally discovered by Oxford et al¹ in 1939 as a metabolic product of *Penicillium griseofulvum* Dierck X. The chemical structure of this metabolite was found to be C₁₇H₁₇O₆Cl by Grove et al² in 1951. Griseofulvin is also produced by other strains of penicillia, more specifically those that produce abundant conidia and few sclerotia.³

Griseofulvin was originally studied as an agricultural antifungal agent, and in 1958 Gentles⁴ reported the successful use of orally administered griseofulvin in treating guinea pigs infected with *Microsporum canis* and *Trichophyton mentagrophytes*. The first clinical report on the use of griseofulvin in man was read by Riehl⁵ in Vienna in November, 1958.

Griseofulvin has been reported by Paget and Walpole⁶ to have effects on mitosis resembling those of colchicine when given in large doses to rats intraperitoneally or intravenously, these effects being particularly pronounced on bone marrow and seminal epithelium. It has been noted, however, that other drugs that have been used clinically will produce similar pathological changes in animals if given by parenteral routes.⁷ A possible explanation for the histological changes in seminal epithelium in rats is the anatomical fact that there is direct continuity between the peritoneal cavity and the scrotum so that the sensitive germinative epithelium is bathed in any agent that is injected intraperitoneally.

In a recent clinical study by Blank and Roth,⁸ in which blood surveys and sternal marrow examinations were performed on patients receiving griseofulvin, no evidence of antimetabolic effect was demonstrated and toxic reactions were minimal.

Griseofulvin is a fungistatic substance inhibiting growth at concentrations far below those needed to produce a rapid killing effect. It causes fungi to produce short, stunted germ tubes whose development ceases at an early stage. In the continuous process of desquamation and re-epithelization, these fungal elements are cast off with cellular debris and the new epithelial elements develop and mature without infection. The length of treatment is related, therefore, to the epithelial structure infected, e.g., glabrous skin is cleared of fungi faster than hair structures and the latter are rendered fungus free faster than the slower-growing nails.

Pardo-Castello⁹ concluded that griseofulvin had no fungistatic effect when used locally in cream, ointment, alcoholic solution, or powder form.

CLINICAL STUDIES

Our report deals with laboratory and clinical evaluations in 32 patients, 24 men

* The griseofulvin used in this study was supplied by Dr. Louis Parrish of Ayerst Laboratories.

TABLE I
Dermatomycoses in 32 Patients

Type of involvement	No. of patients	<i>T. rubrum</i>	<i>T. mentagrophytes</i>	Contaminants
Onychomycosis	16	10	0	6
Tinea pedis	8	3	3	2
Tinea corporis and cruris	7	3	1	3
Actinomycosis	1			

and 8 women, with various dermatomycoses who received orally administered griseofulvin. No patient received any form of local treatment. All the patients have been under treatment for at least a month and some as long as four months. The diagnosis was established by positive potassium hydroxide preparations of specimens obtained from involved skin or nails. Cultures were made on Sabouraud's agar medium. On each return visit these studies were repeated, and as treatment progressed it became increasingly difficult to obtain positive cultures, although potassium hydroxide preparations usually remained positive until the scaling had disappeared or the affected nail had grown out completely. In several instances, although the potassium hydroxide examination was diagnostic, identifiable cultures were never obtained due to repeated overgrowth by contaminants (table I).

The duration of infection varied from 20 years to three months. The clinical response was not related to the chronicity of the disease, with old infections responding as quickly as relatively recent ones.

Dosage. The dosage schedule has been, in the main, 2.0 Gm. daily for the first eight weeks of treatment and 1.0 Gm. daily thereafter or until treatment was terminated. It appears that the accepted therapeutic dosage will be 1.0 Gm. daily. All patients treated showed improvement.

Toxicity Studies. Before the start of treatment, the following laboratory studies were obtained: urinalysis, complete blood counts, sperm counts, and liver function studies including cephalin flocculation, thymol turbidity, bilirubin, alkaline phosphatase, and Bromsulphalein retention. These studies were repeated two weeks and four weeks after the start of therapy and every four weeks thereafter, so that more than 140 batteries of tests were done in the 32 patients.

Results. There were no changes in the urinary findings or alterations in the hemoglobin levels or erythrocyte counts. In 3 patients the level of the leukocyte count fell to 4500 once during treatment but rose to normal without discontinuance of the medication. In 1 patient the leukocyte count was less than 5000 at the start of treatment but returned to the normal range during treatment. Another patient had a drop in leukocyte count to 3900, which returned to normal levels shortly after treatment was discontinued and not restarted because of an unrelated medical problem. Eosinophilia was not present in any of the 32 patients nor was there any consistent change in the differential distribution of the polymorphonuclear cells and the lymphocytes.

None of the patients had a demonstrable change in hepatic function as measured by Bromsulphalein retention, alkaline phosphatase, bilirubin, thymol turbidity, and cephalin flocculation.

TABLE II
Response in Sperm Count

	Sperm count in millions with % motile forms									
	Case 1	Case 4	Case 5	Case 9	Case 10	Case 17	Case 21	Case 24	Case 28	Case 29
Start of therapy	129.4 45%	150.0 42%	21.4 60%	88.5 80%		33.9 78%	8.1 68%	21.5 62%		187.5 82%
2 weeks	68.5 53%	88.4 30%	20.4 34%	133.4 84%		12.5 48%	114.4 65%	39.8 48%	120.0 65%	
4 weeks	32.0 47%	140.0 35%	32.9 44%	66.0 65%	50.0 23%	10.0 48%	80.5 65%		40.5 57%	126.8 54%
8 weeks	54.0 45%	170.0 56%	106.3 —	165.6 36%		12.0 44%				
12 weeks	75.5 60%			98.0 38%	69.3 21%					
16 weeks	91.5 57%			56.0 66%						

The sperm counts showed a variable response, with some increasing and some decreasing; they are recorded in table II.

No cutaneous allergic phenomena were seen, and 2 patients who had a history of previous penicillin sensitivity had no adverse reaction to griseofulvin.

Subjective Effects. Three patients experienced lightheadedness or a dizzy sensation when taking the medication in a fasting state. Two patients complained of headaches shortly after the start of therapy but these disappeared even though treatment was continued. One patient developed diarrhea after being on the medication for eight weeks and this disappeared when the drug was stopped only to recur when therapy was restarted.



FIG. 1 (left). A 44 year old male with onychomycosis of three years' duration due to *T. rubrum*.

FIG. 2 (right). Same patient after 14 weeks of treatment.

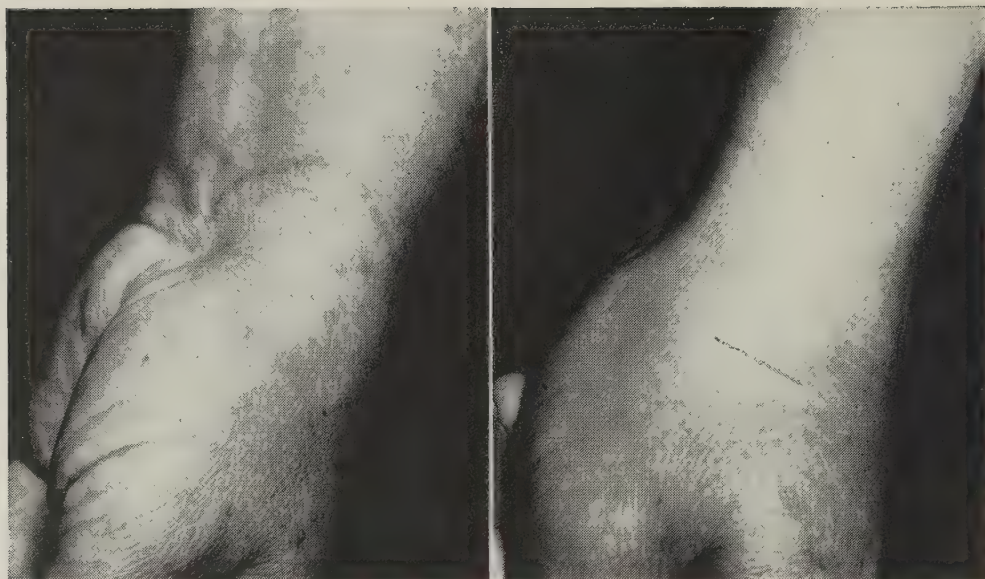


FIG. 3 (left). A 41 year old female with annular lesion due to *T. mentagrophytes* of two years' duration before treatment.

FIG. 4 (right). Same patient after two weeks' griseofulvin. Potassium hydroxide test negative.

Effect on Other Skin Disorders. Coincidental dermatitis herpetiformis in 2 patients was not changed, and 2 patients with concurrent tinea versicolor (*Malassezia furfur*) were not improved by griseofulvin therapy. It has been shown already that *Malassezia furfur* and *Candida albicans* infections do not respond to griseofulvin administration, whereas infections due to the various species of *Microsporum* and *Trichophyton* are susceptible.



FIG. 5 (left). A 21 year old male with extensive tinea corporis of 18 months' duration due to *T. mentagrophytes*, before treatment.

FIG. 6 (right). Same patient after four weeks of griseofulvin. Potassium hydroxide test negative.

Patient Response. Patients with severe pruritus reported disappearance of this symptom within two to six days after starting therapy. The most appreciative patients were those who had long-standing tinea corporis and tinea cruris. Patients with onychomycosis, an entity refractory to all forms of therapy prior to the advent of griseofulvin, were most pleased to see and follow the growth of new, normal-appearing nails (figs. 1-6).

RESULTS

Tinea Corporis, Tinea Cruris, and Tinea Pedis. Patients with involvement of the glabrous skin only were kept on the medication for two weeks after the potassium hydroxide preparations were negative; total therapy has ranged from four to eight weeks. Two of the 8 patients who had tinea pedis have had a recurrence of the infection; both were caused by *Trichophyton rubrum*. One patient had been treated five weeks and the potassium hydroxide test became positive again 11 weeks after medication was stopped. The second patient had eight weeks of medication and the potassium hydroxide test became positive again 12 weeks after cessation of treatment. Whether recurrence was due to reappearance of a subclinical infection or to reinfection from contaminated footwear is not known.

Onychomycosis. All patients with nail involvement are still under treatment, and those who have been treated for nearly four months have had 70 to 90 per cent improvement as evidenced by the presence of normal-appearing nail substance. The possibilities of relapse or reinfection are not known at this time. In onychomycosis it usually takes four to six weeks before improvement is noticeable; nails of the fingers have usually shown regrowth of normal substance before those of the toes in patients with involvement of both.

Actinomycosis. One patient with *Actinomyces bovis* infection of the tongue and floor of the mouth has been on 2.0 Gm. of griseofulvin daily for 16 weeks and now has less induration and fixation of the structures involved. It is still too early to draw any conclusions as to the value of griseofulvin in this disease, since any treatment for actinomycosis is a prolonged one.

SUMMARY

Griseofulvin, an orally administered metabolic product of certain strains of penicillia, was found to be effective in treating superficial mycoses due to *T. rubrum* and *T. mentagrophytes* and possibly *Actinomyces bovis*, a deep mycosis.

Response to treatment was not related to the length of time that the fungus disease had been present. Pruritus usually disappeared a few days after treatment was started. Griseofulvin is of apparent low toxicity in dosages of 1.0 to 2.0 Gm. daily, as evidenced by clinical observation and extensive laboratory studies.

There was no change in urinalyses, hemoglobin levels, or differential counts. A decrease in total leukocyte count occurred in 4 patients but the count returned to normal though the medication was continued in 3. In the fourth patient therapy was stopped due to extraneous circumstances. There was no alteration in hepatic function.

There was no constant trend in the variability of the sperm counts.

Symptoms of headache and vertigo were infrequently encountered and were of a transitory nature in all patients. Diarrhea was significant in only 1 patient.

Tinea versicolor infections are not improved by griseofulvin.

Further experience is necessary to study the problem of relapsing or recurring infections and to determine the end point of the treatment period. Therapy should not be started unless a positive potassium hydroxide preparation is demonstrated and cultures on Sabouraud's media or the clinical appearance and history demonstrate a fungus infection that will respond to griseofulvin.

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Dermatomycoses Treated with Griseofulvin

NORMAN J. GOLDFARB, STANLEY A. ROSENTHAL, ARTHUR BACK, ALEXANDER BOROTA, LIONEL DICK, DOMENICA FURNARI, AND CATHERINE FRASER

Department of Dermatology and Syphilology, New York University—Post Graduate Medical School (Dr. Marion B. Sulzberger, Chairman) and the Skin and Cancer Unit of University Hospital, New York, N. Y.

During 1958, several dermatological departments in various parts of the world initiated clinical studies on the treatment of human dermatomycoses with griseofulvin. The preliminary papers of Riehl,¹ Williams et al.,² and Blank and Roth,³ appearing within a short time of each other, have demonstrated the remarkable efficacy of this oral antibiotic in the treatment of many superficial mycotic infections. Recent work from Mexico^{4,5} has suggested the possible value of griseofulvin in some of the deep mycotic diseases, namely, nocardiosis and sporotrichosis.

The clinical effectiveness of griseofulvin is combined with a conspicuous lack of serious subjective side reactions. Laboratory studies, such as blood examination, liver and kidney function tests, on persons receiving griseofulvin therapy have showed no deviation from the normal range.³

The purpose of this paper is to present the results of our studies with griseofulvin on human mycoses and to compare this drug to topical antifungal medications commonly used.

METHOD

A total of 137 persons with various mycological afflictions, treated orally with griseofulvin, have been studied over a period of several weeks to 10 months. No other therapy was permitted. The vast majority of patients were maintained on a dosage of 1 Gm. a day. However, in specific cases, dosages ranging from 0.25 to 3 Gm./day were employed. In addition to clinical observations, the patients were subjected to mycologic, liver and kidney function, blood and urine examinations before and during the course of therapy. In several instances semen specimens were subjected to analysis.

For the purpose of clarity, the clinical and mycological findings will be discussed according to the sites and structures involved.

Since climate is an important factor affecting therapeutic results, we wish to emphasize that our studies commenced in the winter months and extended into a hot, humid summer in New York City.

CLINICAL RESULTS

Tinea Corporis. Twenty-nine patients were adequately followed under daily therapy of 0.75 to 1.0 Gm. of griseofulvin over a range of 1 to 20 weeks.

During the first week of therapy, 83 per cent experienced subjective and objective improvement. Pruritus diminished markedly by the second to third day while erythema subsided frequently in three to five days of therapy.

Although one patient was clinically cured after seven days of therapy, the ma-

jority of patients (69 per cent) were clinically cured during the second and third week of treatment. By the ninth week, all patients with glabrous lesions without follicular involvement were clinically cured. However, when follicular involvement was present, response to therapy was slower, with clinical cure being achieved during the second to fourth month of therapy.

Twenty subjects were followed mycologically for as long as five months. Prior to therapy, *Trichophyton rubrum* was isolated from 18, *Trichophyton tonsurans* from 1 and 1 subject failed to yield any culture growths although microscopically positive. Mycologic examinations were taken at varying intervals starting as early as one week after commencing griseofulvin treatment. Eight patients became mycologically negative in one to six weeks of treatment. However, of the 12 others who were mycologically positive at six weeks (by potassium hydroxide examination or by culture), only 3 patients were positive as long as four to five months on therapy. It is important to note that 2 of these 3 patients had follicular involvement.

Tinea Cruris. In 20 subjects with mycotic infections of the groins, clinical response at this site was most rapid and favorable. Subjective and objective signs of improvement were noted in all patients within 2 to 14 days. Pigmentation appeared in most sites following fading of erythema.

Clinical cure was achieved in 50 per cent of subjects in one to three weeks. The remainder were clinically cured by the seventh week of therapy.

Nine subjects were followed mycologically over a period of two months. Prior to therapy, cultures of *T. rubrum* were isolated from 6, while the remaining 3, although culturally negative, were microscopically positive. Mycologic examinations taken at varying intervals, starting as early as one week after initiating therapy, failed to reveal any culture growths of *T. rubrum*. In 1 patient a change in mycotic flora from *T. rubrum* to *Candida albicans* was noted. Microscopic examination for fungi became negative one to three weeks after therapy commenced.

Tinea Manuum (Palms). Out of a total of 34, there was adequate observation of 29 subjects over a period varying from several weeks to five months. Daily dosage of griseofulvin, administered orally, was usually at a level of 1.0 Gm.

Twenty-one patients (72 per cent) noted early improvement by the end of the second week of therapy. Pruritus usually diminished in the second to fourth days, with erythema decreasing by the seventh day. Scaling was less marked by the second week. Palms were noted to be smoother in the third and fourth week. Return to normal sweating of palms (where previously absent) was noted during the third to sixth weeks after therapy was commenced. All patients displayed some degree of improvement by the tenth week.

Clinical cure was obtained in 24 patients (82 per cent) with the majority of cures occurring between the third to ninth week of therapy. Four patients, although improved, failed to achieve cure even after five months of treatment.

Twelve patients were mycologically followed. Six of these were negative (both potassium hydroxide and culture) by the sixth week. The other 6 patients were mycologically positive at the end of the sixth week, but 3 of these finally became negative by the fifteenth week of treatment.

Tinea Pedis. Sixty-six persons with tinea pedis have been treated with griseofulvin, usually at the level of 1 Gm. daily. Thirty-nine (58 per cent) persons were clinically improved by the end of two weeks of therapy. All others showed the first

objective signs of improvement in periods ranging from three weeks to two months. These signs were decreases in scaliness, erythema, interdigital maceration, and a diminution of hyperkeratosis. Pruritus, where previously present, was markedly decreased after two to four days of treatment. A few patients reported less itching in the first day of therapy.

However, clinical cure was achieved in only 38 patients (58 per cent) within periods ranging from 2 to 18 weeks with the vast majority of these being cured by the tenth week.

It is to be noted that 28 of the 66 persons, who were under treatment for as long as five months, never achieved complete cure, although marked improvement was noted in all.

Of 42 patients followed mycologically, all were infected with *T. rubrum*, except for 4 cases with *Trichophyton mentagrophytes*. No patient became mycologically negative prior to the third week of therapy. During the fourth week of treatment 9 of 42 became negative; by the twentieth week an additional 9 patients were negative. Twenty subjects remained mycologically positive while under therapy for one to four months.

In 2 patients a change in mycotic flora from *T. mentagrophytes* and *T. rubrum* to *C. albicans* was noted.

Tinea Capitis. Groups of children with tinea capitis were treated with daily doses of griseofulvin ranging from 0.25 to 1.0 Gm.

In the 0.25 Gm. daily dose level, 5 patients were adequately observed. The individual total dose was less than 10 Gm., with earliest signs of improvement noted after two weeks of therapy. However, clinical and mycologic cure was achieved in only 2 of these 5 cases.

At the 0.5 Gm. daily dose level, 6 patients were adequately observed. The total individual dose varied between 10 and 35 Gm. The earliest signs of improvements were mainly noted between 7 and 28 days of therapy. These were a change of fluorescence from the typical green color to a silver grey in some of the *Microsporum* infections, absence of new broken hairs, disappearance of black dots on scalp, and less scaling of scalp. In this group 4 patients (67 per cent) achieved clinical and mycologic cure.

At the 1.0 Gm. daily dose level, a total of 6 patients were adequately studied. The individual total dosage varied between 14 and 21 Gm. administered over a two to four week period. Earliest signs of improvement were the same as observed in 0.5 Gm. schedule. As for those on 1.0 Gm. daily dose, however, 5 cases (83 per cent) achieved clinical and mycologic cure. The 1 failure had been treated for a period of 14 days only, while the others who were cured had 21 to 28 days of therapy.

In 4 patients receiving one initial dose only, varying from 1 to 3 Gm. of griseofulvin, no cures were noted.

In the four dosage schedules discussed there were 14 patients, several of whom received more than one course of therapy.

Of the 14 patients with tinea capitis, there were 8 cases of *Microsporum audouinii*, 3 cases of *Microsporum canis*, and 3 cases of *T. tonsurans*. No resistance to therapy was noted in any of the species. Their response to therapy was alike, depending on daily dosage and length of treatments.

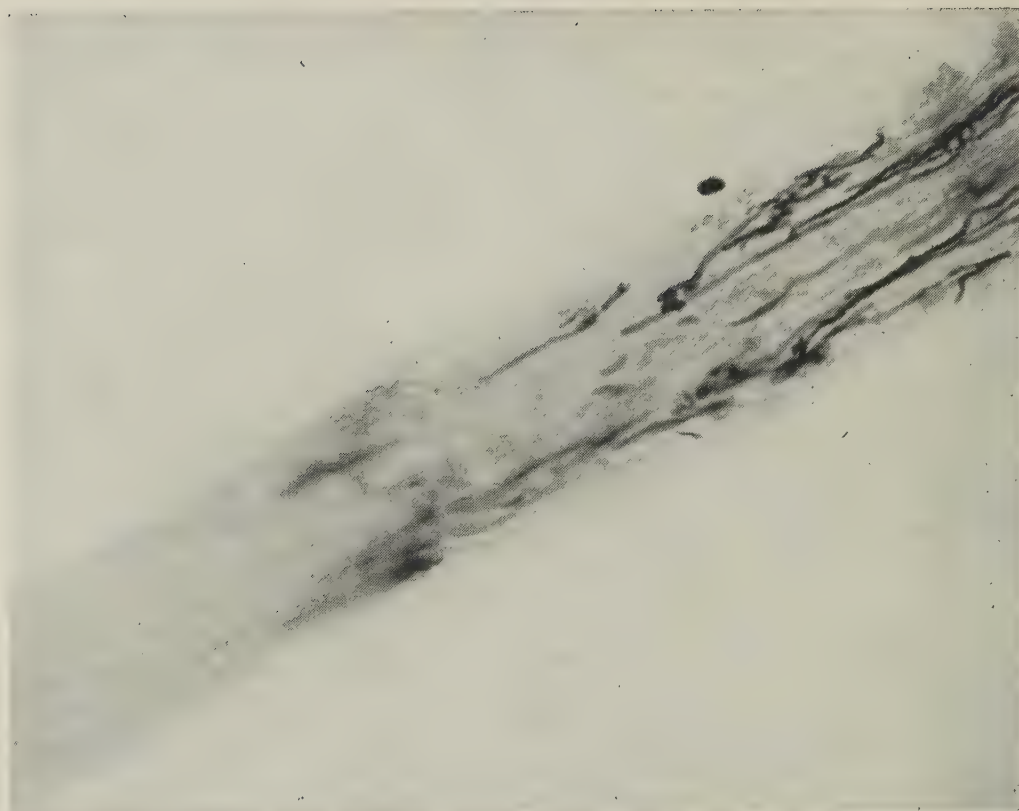


FIG. 1. Hotchkiss-McManus stain of hair from patient with *M. canis* infection of scalp after six weeks of griseofulvin therapy, 1 Gm./day. (X 359.)

As was expected in hard keratotic structure, such as hair, a definite band could be noticed dividing the infected portion from that of the newly growing treated hair. Hotchkiss-McManus stains of treated hairs demonstrate this point clearly (fig. 1).

Although clinical cure had been obtained, there were 4 cases where mycologic findings remained positive. All eventually suffered a recurrence and were re-treated successfully at one of the daily dosage schedules mentioned.

Finger Nails. A total of 39 patients with onychomycosis of the hands due to *T. rubrum* were treated with oral griseofulvin, 1.0 Gm. daily, over a period ranging from several weeks to eight months.

Thirty-six (92 per cent) displayed the earliest emergence of new normal nail growth between the first to tenth weeks of therapy. This was often accompanied by less periungual scaling, less subungual hyperkeratosis, less onycholysis, and cleaner appearance of the distorted nail plate.

However, of this group, only 16 patients (41 per cent) achieved cure during this period of observation. This occurred during the second to sixth month of treatment. Twenty-two patients showed varying degrees of continuing improvement, depending upon length of therapy.

One patient, after satisfactory initial response, failed to continue his improvement although maintained on therapy.

TABLE I
Clinical Improvement on Griseofulvin Therapy
(1 Gm. daily)

Site	No. of cases	Clinically improved	Average time for earliest improvement	
			No. weeks	% patients
Glabrous skin	24	24	1	83
Crural area	20	20	1	80
Glabrous skin (follicular)	5	5	2	80
Palms	29	29	2	72
Soles	66	66	2	59
Scalp and scalp hair	14	14	3	(0.5-1 Gm.) 75
			4	(0.25 Gm.) 60
Finger nails	39	39	4	53
Toe nails	58	46	7	60

In some instances, continued improvement and even clinical cure of finger nails was apparent after therapy had been discontinued.

Toe Nails. A total of 58 patients with *T. rubrum* infections of the toe nails have been studied and adequately followed. Due to the slow growth rate characteristic of this keratinous structure, the clinical response was expectedly less dramatic compared to the other sites.

Forty-five of 57 patients (79 per cent) displayed the earliest signs of new nail formation in one to three months under a daily dose of 0.75 to 1.0 Gm. of griseofulvin.

In a group of 12 patients observed under therapy over a period of four and one half to six months, new nail formation was found to range from 30 to 70 per cent of the normal nail length. However, only 2 patients achieved a cure after three and one half and five months, respectively, of therapy.

It is in this group that, following slight improvement, there were several instances of no further progress in spite of continued therapy over several months.

TABLE II
Clinical Cure on Griseofulvin Therapy (1 Gm. daily)

Site	No. of cases	Clinically cured		Average time for clinical cure (weeks)
		No.	%	
Glabrous skin	24	24	100	3
Crural area	20	20	100	3-4
Glabrous skin (follicular)	5	5	100	11
Palms	29	24	82	5-6
Soles	66	38	58	8
Scalp and scalp hair	14	12	85	8 (0.5-1 Gm.)
				5-6 (0.25 Gm.)
Finger nails	39	16	41	16
Toe nails	58	2	3.5	—

TABLE III

Mycologic Findings at Time of Clinical Cure

Site.	No. of clinical cures	No. of clinical cures with adequate mycological follow-up	Clinically and mycologically cured*	Healthy carrier state (clinically cured but mycologically positive)
Glabrous skin	26	13	10	3
Crural area	20	8	6	2
Glabrous skin (follicular)	5	5	1	4
Palms	24	10	8	2
Soles	38	20	13	7
Scalp and scalp hair	12	12	8	4
Total	125	68	46	22

* Cases that were clinically apparently cured and were repeatedly negative mycologically (microscopically with potassium hydroxide preparations and by culture).

It is difficult to analyze the mycological data obtained from nail sites, since the infected nail portions were adjacent to the apparently normal new increments steadily appearing under treatment.

Summary of Clinical Data. The data concerning the time required for clinical improvement and clinical cure, as well as mycologic findings at the time of clinical cure, are summarized in tables I, II, and III.

MANAGEMENT

Those patients requiring long-term therapy were treated with an oral daily dose of 1.0 Gm. until satisfactory improvement was obtained. Then a maintenance dose was determined by the clinical progress noted and the structure involved. For glabrous skin and crural areas generally 0.5 Gm. daily was satisfactory. Palms and soles usually required 0.5 to 0.75 Gm. daily maintenance dose. Finger nails would progress satisfactorily with 0.75 Gm. daily while toe nails demanded 1.0 Gm. daily for continued steady improvement.

Although cures were obtained among the tinea capitis patients at each daily dose level of 0.25, 0.5, and 1.0 Gm., the greatest cure rate was noted in those treated at 1.0 Gm. daily for a three to four week period.

LABORATORY DATA

Complete blood counts were found to vary within normal limits during the course of therapy. No abnormalities were detected in liver or kidney function tests.

Semen counts were done on several patients and found within normal values during and after completion of therapy.

Thirty patients had received total doses of griseofulvin between 50 to 130 Gm. without evidence of toxicity.

UNDESIRABLE SIDE REACTIONS

These were substantially few in number and of minor consequence. The most

frequent complaint was that of occipital headache in 24 of 137 patients treated. The headache was transient in character and by far the greater number disappeared during the second week of therapy. Administration of aspirin relieved this distress to a great extent.

Fatigue and drowsiness were noted in 10 patients. Diarrhea was noted in 7 patients; nausea in 6; transient pruritus in 3; transient vertigo in 2; generalized maculopapular eruption (resembling a drug eruption) in 2 patients; and photosensitivity in 1. Only occasionally was it necessary to discontinue therapy because of either severe headache, persistent diarrhea, or extreme fatigue.

DISCUSSION

Following initial improvement under griseofulvin therapy, a few of the patients experienced a sudden intertriginous dermatitis of groins and/or toe webs. Potassium hydroxide preparations of skin scrapings from these areas were positive for hyphae. However, culture growths did not reveal the original etiologic isolate but rather *C. albicans*. It is possible that with the original dermatophyte inhibited, *C. albicans* had an opportunity to grow.

It is to be noted that in the patients achieving clinical cure with griseofulvin, one third were still mycologically positive. These patients are regarded as being in the "healthy carrier state." It would be most likely that the combination of local desquamative therapy with oral griseofulvin might lead to a greater number and more rapid attainment of clinical and mycologic cures.

Successful treatment with oral griseofulvin is most dramatic in regard to onychomycosis and tinea capitis. Previously onychomycosis remained recalcitrant to all forms of local therapy. Even surgical removal of nails was connected with a high rate of recurrence. In fact some dermatologists would not encourage treatment. We have now witnessed the regrowth of normal nail from the matrix in patients under griseofulvin therapy. Although the time required for cure of nails is several months, our patients have been extremely happy with the cosmetic improvement.

With regard to tinea capitis of the non-inflammatory type, it is well known that prepuberty cure usually requires roentgenographic or thallium depilation. In some instances spontaneous cure has occurred, usually by development of inflammatory reaction, but this is not common. Lately the tendency for using less roentgenotherapy in benign lesions in order to reduce exposure to radiation has restricted even this valuable tool in treatment of tinea capitis. It is therefore fortunate that griseofulvin has made its clinical debut.

In cases of tinea pedis and tinea manuum response to local soothing and desquamative medication has brought relief of symptoms usually. However, many of these inveterate cases have frequently recurred with the onset of warm weather. The use of griseofulvin in these patients uniformly brings relief of symptoms quite rapidly. Of special note is that the hard keratin masses of the soles gradually diminished after two to three months of griseofulvin therapy.

Follicular glabrous lesions in the past have required prolonged local therapy. At times even surgical curettage was required for persistent deeper lesions. With griseofulvin symptomatic relief was quickly attained but the length of time required for cure may extend over a period of two to four months.

Lesions of the glabrous skin and crural areas, when erythematous and of inflammatory nature, in the past usually responded fairly well to local soothing measures. No particular advantage could be noted in favor of griseofulvin except for the ease of administration associated with the use of an oral medication. However, the dry type of fungous infection (usually *T. rubrum*) was often resistant to therapy. Irritations from strong external medications were commonly encountered. Recurrence rate was high. With griseofulvin, these lesions respond quickly and uniformly with relief of itching noticed as early as the first or second day.

SUMMARY

A total of 137 patients with various dermatomycoses have been treated with oral griseofulvin generally at a level of 1.0 Gm. daily and observed over a period of several weeks to 10 months. Response to this therapy was rapid and most efficacious especially in those cases known to be of a previously inveterate nature, such as non-inflammatory tinea capitis and onychomycosis.

Inflammatory superficial ringworm infections responded equally well to local soothing measures or oral griseofulvin.

The hyperkeratotic type and non-inflammatory type of dermatomycoses responded better to oral griseofulvin subjectively and objectively in comparison with local medication alone.

Griseofulvin appears to be a major advance in dermatologic therapy of superficial ringworm infections.

ACKNOWLEDGMENT

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Griseofulvin Therapy of Tinea Capitis

HOWARD T. BEHRMAN, IRWIN I. LUBOWE, EDWARD H. MANDEL,
AND JOSEPH L. MORSE

*Department of Dermatology, New York Medical College,
Metropolitan Medical Center, New York, N. Y.*

Griseofulvin* is a new antifungal antibiotic, which is a fermentation product of several species of *Penicillium*¹ (*Penicillium griseofulvum*, *Penicillium patulum*, *Penicillium janczewski*, and others). Unlike penicillin, it is not bactericidal; furthermore, it differs immunologically from penicillin and has been given to patients known to be sensitive to penicillin without reaction. Although isolated as far back as 1939, this drug was not employed therapeutically until 1958, when Gentles² found it effective in the treatment of experimental ringworm in animals. Subsequent studies³⁻⁶ rapidly proved its efficacy in a varied group of dermatomycoses, including certain types of ringworm of the scalp, body, and nails. Certain fungi, such as the *Microsporum*, *Epidermophyton*, and *Trichophyton* genera, respond well, whereas yeasts such as *Monilia* and deep-seated mycoses such as actinomycosis and histoplasmosis are unaffected.

Griseofulvin is absorbed from the gastrointestinal tract and deposited in the keratin of the skin, hair, and nails. When an effective concentration has been obtained, the drug is fungistatic and not fungicidal. It inhibits the growth of susceptible fungi, especially the growing tips of the terminal hyphae, but they remain viable within the infected tissues. Therefore, treatment with griseofulvin must be continued until the infected hairs, skin areas, or nails are removed either by natural processes or artificial measures.

The purpose of this study is to delineate a long-range method of control of tinea capitis with the new antifungal antibiotic, griseofulvin. These therapeutic aims were subdivided as follows: (1) the determination of the efficacy of griseofulvin in tinea capitis; (2) the finding of minimal, maximal, and optimum dosage levels; (3) the incidence of recurrence and reinfection; (4) the necessity for other procedures or therapeutic modalities; (5) necessary public health measures in conjunction with the use of this drug; and (6) the incidence of toxicity and side reactions.

The classification of these factors will require a very considerable period of time and further observation, and this report is offered merely as a preliminary study.

MATERIAL AND METHODS

As of the present writing, over 100 children with tinea capitis are under treatment with griseofulvin. The drug has been administered in a daily dosages ranging from 7 to 40 mg./lb. Many different treatment regimens are under evaluation, and these vary from minute to massive dosage, from short to medium and long periods of administration, and from regular to intermittent dosage schedules.

Wherever possible, the children are examined at weekly intervals. Blood counts

* The trade name of the Schering Corp. for griseofulvin is Fulvicin.

and urinalyses are performed before, during, and after therapy. Wood's light examinations, microscopic examinations of the hairs and scales (in potassium hydroxide), and cultural studies are performed at regular intervals. The criteria of cure include at least two negative Wood's light examinations in conjunction with two negative cultures (Sabouraud's medium or some of the newer selective isolation media) at intervals of two weeks. It is essential to check negative cultures in tubed medium by inoculating hair stubs and scalp scrapings into Petri dishes containing Sabouraud's medium, inasmuch as residual infectious material may still be present in some of this nonfluorescent or slightly fluorescent residue.

RESULTS

Cultural studies revealed that more than 95 per cent of the infections in this group were due to *Microsporum audouini*. For all practical purposes, therefore, this project is a study of the effects of griseofulvin on this resistant form of ringworm of the scalp.

Over 100 children are under treatment with this drug, and approximately half are considered cured by our criteria. It is still too early to discuss the question of treatment failure, recurrence, and reinfection. The patients' ages ranged from 1 to 12 years, and the scalp involvement varied from a few infected hairs to many large patches. The vast majority of children are Negroes, and in many instances the infection has been present for several years, despite various forms of therapy.

It was obvious, after several months' time, that a daily dosage of 10 mg./lb. for four to six weeks would cure most cases, provided the hair was either cut short or shaved off after four weeks. However, 1 child was cured with a dosage of 7 mg./lb. daily for three days, and another had either a recurrence or reinfection five weeks after apparent cure with a dosage of 13 mg./lb. daily for 42 days.

The fluorescence of the infected hairs under the Wood's light underwent a gradual change from week to week. The fluorescences rarely disappeared completely until at least four to eight weeks had elapsed (after institution of therapy) unless, of course, the hair was cut short. The usual bright green color of the infected hair went from bright green to dull green to bright white and, finally, dull white. The infected hairs showed a sharp transition between the fluorescent and normal portions of the hair shaft corresponding with the growth of the hair after the institution of griseofulvin therapy. As long as fluorescence persisted, positive cultures could be obtained from the fluorescent portion of the shaft.

Blank and Roth⁵ have demonstrated that the degree of growth of new hair can be readily determined by placing a hair plucked from the head of a patient under treatment on an agar plate seeded with a sensitive organism, such as *Trichophyton rubrum*. The new portion of the hair contains active griseofulvin, which produces inhibition of the organism. The old portion of the hair contains no antibiotic and therefore does not become surrounded by a clear inhibiting zone.

The earliest negative culture was obtained four weeks after inception of therapy, but in one instance did not occur until 13 weeks later. The usual period was approximately eight weeks, and, in most of these cases, griseofulvin therapy had been terminated at the end the fourth week. However, some patients still under treatment for even longer periods of time are still culture positive.

The urinary and blood findings disclosed no significant changes. One child developed a slight intestinal upset, which disappeared despite continuance of therapy. Several also complained of a mild headache for a few days. According to the mother of another child, a so-called "poor eater," his appetite improved and he gained weight rapidly while taking the drug. Two children who developed chickenpox while their cultures were still positive and their hairs still fluorescent showed a rapid reversal of these findings to negative. This may have been coincidental. Several children also developed a papulopustular, follicular infection in the involved areas of the scalp after one to two weeks of therapy. This infection subsided while still on griseofulvin therapy, with the addition of wet dressings and an antibiotic ointment locally.

COMMENT

Inasmuch as the majority of these patients are still under long-range observation and treatment, a tabulation and specific description of the cases has been deliberately omitted. However, certain observations can be made from the data at hand as well as from that of previously published studies. It is apparent that a daily dosage of 10 mg. of griseofulvin per lb. of body weight for a four week period of time will cure most cases of ringworm of the scalp, provided the hair is either shaved or cut off below the fluorescent level at that time. It must be recognized that this drug is fungistatic and not fungicidal and that viable fungi may still be found in persistent but weakly fluorescent hairs. Such hairs are still present four weeks after the institution of therapy and may even persist for 12 weeks or longer.

We have observed that after three or four weeks of griseofulvin therapy, the diseased portion of the hair often bends away from the normal shaft, producing a comma-shaped hair. This bent portion breaks off easily and may be a source of reinfection or spread to other children.

Cultures from these hairs may still show active growth of the organism. Accordingly, from a public health standpoint and especially in areas where laboratory facilities are inadequate and follow-up controls are limited, patients should receive an appropriate initial therapeutic dosage and must have a close hair cut or shave at the end of the fourth week. Frequent shampoos are also advisable from a personal and public health standpoint. In many instances, these patients come from very low income family groups and health control is practically nil. It should be recognized that some of these patients will report to the clinic for just a single visit. While the importance of observation and therapy may be emphasized to the parent, the patient may still disappear. For this reason we are attempting to find an adequate dosage schedule and routine from the standpoint of protection of the community. The four week schedule and hair shave already discussed may be considered adequate at the present time. However, it should be recognized that this is not the optimum approach. We have seen 1 child cured with a dosage of one tablet daily for only three days (only 7 mg./lb. daily for a total dosage of 21 mg./lb.—750 mg. altogether). Others have responded to only slightly larger doses. On the other hand, we have a few children still under observation, who have been treated with a daily dosage of 10 to 20 mg. of griseofulvin per lb. for six weeks and longer, and are still Wood's light suspicious and culture positive. Kirk and Ajello,⁶ in their excellent report, stated that 2 of their patients still had positive cultures despite 77 days

of continuous therapy at 750 mg. (11.8 and 12.8 mg./lb.) per day. When our studies are completed, we may find it preferable to administer small doses continuously, for either a shorter or longer period, or to stagger the doses over a much longer period of time, or merely to give a massive dose initially and subsequently shave off the hair. Only time can answer these questions. We may also discover that our entire therapeutic regimen may be influenced by the incidence of recurrences (we had one, and it may have been a reinfection) or by the development of sensitization to this antibiotic as it comes into more popular usage, or by the eventual development of fungal strains resistant to the drug. Again, time alone will provide the answers.

As mentioned previously, we had a very low incidence of toxicity or side reactions of any type. Some children had mild gastrointestinal symptoms, transient headaches, and the like. No unusual hematological or urinary changes were observed.

SUMMARY AND CONCLUSIONS

1. Griseofulvin is by far the most effective drug as yet encountered for the treatment of ringworm of the scalp.

2. Optimum dosage schedules have not been fully determined as yet, but, in the majority of cases, a daily dosage of 10 mg./lb. for four weeks is adequate. However, the hair must be shaved or clipped off at the end of this time and shampooed frequently thereafter.

3. Patients should not be discharged until at least two negative cultures have been obtained at two week intervals. If possible, they should be kept under observation for an additional month with Wood's light examinations and an additional culture taken of any suspicious hair stub or debris observed under this light.

4. The incidence of toxicity and side reactions with this drug is extremely low.

ACKNOWLEDGMENTS

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The Problem of Staphylococcal Infections in the Hospital with Particular Respect to Long-term Medication with Different Antibiotics

GEORG MAURER

Surgical Department, Staedtisches Krankenhaus rechts d. Isar, Munich, Germany

Infections caused by antibiotic-resistant staphylococci are a problem mainly in large hospitals and particularly in surgical wards. Such staphylococcal infections cause a difficult clinical situation more frequently postoperatively, especially in patients whose general condition is already deteriorated. This is particularly true if the infective staphylococci are highly resistant to the antibiotic being given, in which case complications such as wound infections with dehiscence of sutures and sometimes fatal enterocolitis can occur.

Since today, in most hospitals, as well as in general practice, a substantial increase in the number of staphylococci resistant to many or all antibiotics is developing, this problem must be regarded as a matter of greatest importance.

MATERIAL AND METHODS

For the past two years, in our efforts to prevent the problem of staphylococcal infections, we have paid particular attention to the following points: rigid adherence to strict asepsis and general hospital hygiene combined with modern sanitary methods, such as ultraviolet irradiation of mattresses and disinfection of beds and bedding in a special disinfection room; close cooperation with the bacteriological institute and careful control and handling of swabs from the nasopharyngeal and wound area, particularly in cases exposed to bacterial contamination, and bacteriological control of the nursing personnel; planned administration of selected antibiotics, specifically, reduction in the administration of antibiotics as far as possible and limitation of use of only one antibiotic or antibiotic combination at a time. After a certain period of use, we would then institute treatment with another antibiotic, eliminating all others. Thus, this regimen makes use of an alternating medication scheme with complete elimination of certain antibiotics for a certain period of time. The change from one antibiotic to another is made subject to the development of resistance.

A useful dosage scheme is of particular importance, i.e., initial dosages should be as high as possible and continued to normalization of body temperature.

The exclusive use of only one antibiotic or antibiotic combination during a certain period of time offers the following advantages: By changing the antibiotic, an alteration of the type of resistance of the bacterium should be obtained. The reason for this is the fact that resistance is selective for each antibiotic. By alternating antibiotics, each time therapy is changed from one antibiotic to another, if all other measures are rigidly adhered to, then theoretically a change in the type of resistance that bacterial organisms develop can be expected. Another reason for adopting this method was to study the clinical effectiveness of the various anti-

TABLE I
Clinical Results—3161 Patients Treated

	Wound infections, %	Lethal cases in peritonitis, %	Lethal cases of ulcerative colitis
Phase I	1.5	6	0
Phase II	6.02	11	0
Phase III	9.38	17.07	1
Phase IV	7.08	27.69	3
Phase V	5.98	13.2	1

biotics being used exclusively with special respect to the existing staphylococcic population present in the hospital.

Up to now, we have made observations on five phases of the alternating medication scheme. The following antibiotics were used: phase 1—a tetracycline-oleandomycin preparation;* phase 2—penicillin-streptomycin and, in complications in abdominal surgery, chloramphenicol; phase 3—penicillin-streptomycin, a tetracycline-oleandomycin preparation, tetracycline from different manufacturers, erythromycin, chloramphenicol, and, for local application, neomycin and bacitracin; phase 4—chloramphenicol; phase 5—oleandomycin and the fore-mentioned tetracycline-oleandomycin preparation. During the third phase, we purposely disregarded the planned medication system in favor of therapy that allowed the use of several antibiotics according to the usual practice.

RESULTS

During the five phases, 3161 patients received antibiotics. Indications included abdominal surgery, thoracic surgery, septic surgery, and accident surgery (table I).

The following results were obtained (table II).

Phase I (1064 Patients). CLINICAL RESULTS. We noted reduction in the number of wound infections from 15 to 1.5 per cent; a decrease in mortality from peritonitis from 15 to 6 per cent; a decrease in postoperative infections after thoracic surgery from 13 to 5.4 per cent; no case of severe ulcerative colitis.

BACTERIOLOGICAL RESULTS. There was a reduction in the number of streptomycin- and tetracycline-resistant organisms.

Phase II (469 Patients). CLINICAL RESULTS. There was a slight increase in wound infections (to 6.02 per cent); an increase in mortality from peritonitis to 11 per cent; no lethal case of ulcerative colitis.

BACTERIOLOGICAL RESULTS. There was a decrease in sensitivity to tetracycline, a slight increase in resistance to erythromycin and oleandomycin.

Phase III (811 Patients). CLINICAL RESULTS. There was a substantial increase in wound infections (to 9.38 per cent); an increase in severe ulcerative colitis (one death after administration of chloramphenicol); an increase in mortality from peritonitis (to 17.07 per cent).

BACTERIOLOGICAL RESULTS. There was a substantial increase in resistance of

* The trade name of Chas. Pfizer & Co., Inc., for this tetracycline-oleandomycin preparation is Signemycin.

staphylococci to penicillin-streptomycin, tetracycline, erythromycin, and oleandomycin in swabs from the nasopharyngeal and the wound area. Most of the staphylococci were isolated from the two wards where new surgically treated patients were placed. Bacteriological tests confirmed the impression that some of the carriers of these organisms were the physicians and nursing personnel of this ward; on detection of such carriers, they were immediately forbidden to enter these wards.

Phase IV (583 Patients). CLINICAL RESULTS. There was a reduction in wound infections to 7.08 per cent; a significant increase in mortality from peritonitis, to 27.69 per cent; an increase in postoperative ulcerative colitis (3 per cent). On five occasions, chloramphenicol-resistant staphylococci were found in the feces. Three of these patients, with either local or diffuse peritonitis, died.

BACTERIOLOGICAL RESULTS. There was a significant reduction in resistance to penicillin-streptomycin; a very impressive decrease of tetracycline-oleandomycin resistance, particularly in regard to oleandomycin; an increase in resistance to chloramphenicol.

Phase V (234 Patients). CLINICAL RESULTS. There was a reduction of wound infections to 5.98 per cent; a reduction of mortality in peritonitis to 13.2 per cent; one fatal ulcerative colitis after peritonitis.

BACTERIOLOGICAL RESULTS. There was a slight increase of resistance to penicillin-streptomycin and tetracycline-oleandomycin, and a decrease of resistance to chloramphenicol.

DISCUSSION

Based on the results given in the preceding data, the following considerations should be mentioned: The problem of staphylococcal infections in the hospital can be handled only by close cooperation with a bacteriological institute because con-

TABLE II
Resistance of Staphylococcus aureus to Various Antibiotics: Results of Bacteriological Examination of Swabs from the Nasopharyngeal Area

Phase	Antibiotic					
	Penicillin, %	Streptomycin, %	Tetracycline, %	Erythromycin, %	Oleandomycin, %	Chloramphenicol, %
I 1957						
Tetracycline-oleandomycin	34.0	40.0	33.0	11.0	0.9	2.0
II August, 1958						
Penicillin, streptomycin	39.6	30.0	26.9	17.0	15.1	4.9
III December, 1958						
Penicillin, streptomycin, tetracycline-oleandomycin, other tetracyclines, chloramphenicol, erythromycin, and bacitracin plus neomycin sulfate	88.8	82.2	53.3	73.3	73.3	—
IV May, 1959						
Chloramphenicol	55.5	34.9	30.1	3.1	—	28.5
V June to October, 1959						
Oleandomycin, tetracycline-oleandomycin	60.1	56.5	39.3	24.5	24.5	7.7

stant bacteriological control of the ward, as well as of physicians and nursing personnel, is imperative. In the future, our clinical work will be epidemiologically supported by testing the various types of bacteriophages, an investigation method that up to now has been practiced by only one German institute. We expect to discover, by such techniques, bacterial strains of particular virulence and high contagiousness.

We recommend restriction in antibiotic medication, as practiced by us, using dosages as high as is clinically practical. The use of all available antibiotics within an institution, as usually practiced, results in the development of increasing resistance to the majority of antibiotics used. Therefore, the exclusive application of only one antibiotic or one antibiotic combination at a time seems to us to be preferable. Under such circumstances, regaining of sensitivity by the organisms to the antibiotics not used can be expected, at least to a certain extent. Very little success can be expected in regard to organisms regaining penicillin sensitivity since staphylococcal resistance to one broad-spectrum antibiotic is usually also accompanied by resistance to penicillin. In a hospital with the problem of staphylococcal infection, therefore, it is recommended that therapy with this method involve changes from one to another broad-spectrum antibiotic.

According to our experience, particularly in abdominal surgery, antibiotic combinations are useful, for their action is characterized not only by a delayed development of resistance but also by an increased therapeutic effectiveness against the infecting organisms in numerous cases. In addition, we would like to state that, particularly in cases of local or diffused peritonitis, i.e., in serious situations, as broad an antibiotic effect as possible is necessary, since in an appreciable number of cases, mixed infections are present. Especially in abdominal surgery it is imperative to prevent the development of superinfections, since such complications most frequently appear with local or diffused peritonitis and because postoperative ulcerative colitis frequently is caused by resistant staphylococci. Prevention of such complications should be easier to accomplish by using an antibiotic combination. The marked effectiveness of the tetracycline-oleandomycin preparation, for example, is significantly demonstrated by the decreased mortality in ulcerative colitis cases observed during the first and fifth phases when this combination was used. Of particular interest is the fact that the exclusive administration of this preparation gave rise to the best clinical and bacteriological results obtainable.

Beyond the possibility of long-term use of this preparation (increasing resistance was observed only after 10 months of administration), a further advantage is to be seen in comparison with the other medication phases in treatment of peritonitis, incidence of ulcerative colitis, and wound infections.

Change from one antibiotic to another is recommended depending on the bacteriological results observed. The longer the single phases of the alternating antibiotic medication can be extended, the better are the prospects for recurrence of sensitivity to the antibiotics being restricted. When resistance to the antibiotic being used begins to increase, which is usually signaled by the clinical observation that an increase in wound infections, enterocolitis, and severe septic complications is occurring, further prolongation of the antibiotic being used does not seem to be recommended, according to our experience. When such a development takes place, change to another antibiotic is indicated.

SUMMARY

In our opinion the problem of resistant staphylococcal infections in the hospital can be solved only by correlating all the measures described in our study, specifically strict adherence to aseptic techniques and general hospital hygiene, close cooperation of clinicians and bacteriologists, and use of fully effective dosages of single antibiotics or antibiotic preparations administered throughout the hospital generally for the therapy of all infections.

We are of the firm opinion that the use of all available antibiotics at one and the same time within a hospital, with change from one to another if the first does not produce the desired effect rapidly, or even the blind adoption of any and all new antibiotics introduced into medical practice will never provide the answer to the problem of staphylococcal infections in the hospital.

Studies on the Emergence of Oleandomycin-Tetracycline Combination Resistant Strains of *Staphylococci*

A. M. TORRA AND L. SALDAÑA TATE

Clinical and Laboratory Investigation, Hospital Pasteur, Montevideo, Uruguay

Some authors^{1,2} have reported clinical evidence that the combination of oleandomycin-tetracycline delays the emergence of resistance in *Staphylococcus* strains and others have reported a similar delaying action in vitro. We have considered it of interest to study the producing of such delayed resistance in clinical material and to compare the parent drug-sensitive microorganisms with the same microorganisms that are acquiring or have acquired a permanent resistance under a single drug or a combination of the two.

We have found very few oleandomycin-resistant strains in our hospitals where we currently use a combination of oleandomycin-tetracycline for *Staphylococcus* infections. In the last three years, the percentage of resistant strains has increased from 0 to 14.3 per cent. On the other hand, we have learned that in another of our hospitals, where only oleandomycin is used in the treatment of these infections, the percentage of resistant strains is considerably higher.

Carrod³ found an increase of resistance using tetracycline but not when using oleandomycin, repeatedly exposing strains of sensitive staphylococci to an oleandomycin-tetracycline combination. He also pointed out that the addition of tetracycline seems to delay the appearance of resistance to the oleandomycin but that the converse did not seem to happen.

The purpose of this paper is to confirm all these conclusions and to study the changes in the metabolic responses during the emergence of resistance.

MATERIAL AND METHODS

A study was conducted on 14 *Staphylococcus* strains sensitive to both oleandomycin and tetracycline. All the strains had recently been isolated and tested for their biological characteristics (pigment production, enzymatic activity as mentioned elsewhere⁴); dilution tube technique and the bacterial end point were determined by subculture, and to determine the combined effect of the two drugs we used the same technique as Welch et al.⁵

The procedure was then repeated with increasing concentrations (0.062, 0.125, 0.25, 0.50, 1.0, 2.0, 3.9, 7.8, 15.6, 31.2 µg./ml.) of oleandomycin, tetracycline and a 2:1 combination of these.

Determination of Transaminase Activity. The reaction vessel contained 2.2 mg. of washed cells, 0.2 ml. of *M*/2 alphaketoglutarate in *M*/15 phosphate buffer pH 7.3, 0.4 ml. pyridoxal phosphate containing 80 mg./ml., 0.4 ml. *M*/10 solution of the aminodonor in phosphate buffer, 0.5 ml. of the antibiotic solution, and sufficient phosphate buffer to give a final volume of 3 ml. The vessel was incubated in a 37 C. water bath for five hours with constant agitation.

Bacterial cells were removed by centrifuging and transaminase activity estimated in terms of glutamate content in the supernatant fluid.⁶ The glutamic acid was estimated by chromatography.

RESULTS

On determining the transaminase activity, we found that results were rather erratic even with the same strain, so rather than represent the average of several determinations by a single-line curve we used a broad thick band to cover them all. This thick curve, incorporating 127 separate figures eliminates all the individual details allowing us to grasp the most significant facts.

The first contacts with the antibiotic combination induce in the cells a significant decrease of transaminase activity.

The presence of the drug maintains a low figure of transaminase activity showed by the horizontal band.

When the cells have acquired a permanent resistance, the presence of the drug in the medium is unnecessary to maintain the low figure of transaminase activity.

We have found that the permanent resistance of the *Staphylococcus* measured in minimum inhibitory concentration appears after 14 transfers in oleandomycin, after 8 transfers in a tetracycline medium but only after more than 19 transfers in a medium containing the combination of the two drugs.

The initial point of the permanent resistance measured by the permanent decrease of transaminase activity appears after 6 transfers with tetracycline, after 10 transfers with oleandomycin. Using a combination of the two the presence of the drug was still required to maintain a low level of transaminase activity even after 19 transfers, showing a clear delay in the appearance of permanent resistance.

SUMMARY

There is a great variability in the metabolic responses (transaminase) of the same strains of *Staphylococcus* in acquiring resistance. There is a definite delay in the emergence of resistance with the combination of the drugs oleandomycin and tetracycline.

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Development of Resistance Under Control of Antibiotic Consumption

K. KNÖRR

Gynaecological Clinic of the Tuebingen University, Tuebingen, Germany

In previous investigations^{3, 6, 8, 10, 11, 17-19, 21, 22} it was stated that obstetrical infections today are caused practically exclusively by hemolytic staphylococci, also called *Micrococcus pyogenes*. Of particular importance is the fact that these microorganisms develop antibiotic-resistant variants.^{1, 4, 20} This correlation between antibiotic consumption and development of resistance caused us to create certain rules for the clinical use of antibiotics. Thus, one antibiotic was elected for routine use, and a second one was made available as a reserve.

Since January 1, 1957, we have used exclusively only the tetracycline-oleandomycin* combination, with the exception of a few special cases. Chloramphenicol is kept as the reserve. The experimental results of this procedure was followed by determining the appearance of resistant bacteria, a procedure that was carried out in close cooperation with the Hygienic Institute of the Tübingen University. The following findings are the result of 1456 determinations. The test material is derived from environment examinations of the gynecological ward and from swabs of the nursing personnel, newborn infants, and postpartum women.

To start with, the development of resistance against the routinely used tetracycline-oleandomycin is of interest (fig. 1). As was to be expected, the number of resistant bacteria to tetracycline-oleandomycin is slowly increasing. By means of the oleandomycin curve, it can be shown how quickly resistance develops against an antibiotic that was used for the first time in a clinic. During the second year of application, 25 per cent of resistant germs appear. The initially high resistance against tetracycline, as is seen in figure 1, results from a very extended use of tetracycline, chlortetracycline, and oxytetracycline during the years 1952 to 1954. Worth mentioning, however, is the observation that a further increase of resistance against tetracycline-oleandomycin, as was expected for the year 1959, did not occur, and rather a slight, but not significant, decrease of resistance has recently been observed. It may be of further interest to note that the resistance against chloramphenicol, as shown in figure 2, has developed. In spite of the relatively rare administration of chloramphenicol, the resistance has surprisingly already increased to 25 per cent. The extent of decrease of resistance against antibiotics eliminated from therapy is demonstrated by the simultaneous results with penicillin and streptomycin.

The effectiveness of our therapeutic methods becomes significant in view of the incidence of infections in newborn infants and their mothers. The frequency of evidence of mastitis, for example, has been substantially reduced during the last years (fig. 3). The same favorable development is to be seen in infections in newborn infants (table I). However, there is no doubt that the demonstrated decrease in morbidity could only be obtained by simultaneous strict adherence to

* The trade name of Chas. Pfizer & Co. for tetracycline-oleandomycin is Signemycin.

FIG. 1. Resistance of *Micrococcus pyogenes* against tetracycline (— — —), oleandomycin (———), and a tetracycline-oleandomycin combination (— · — · —).

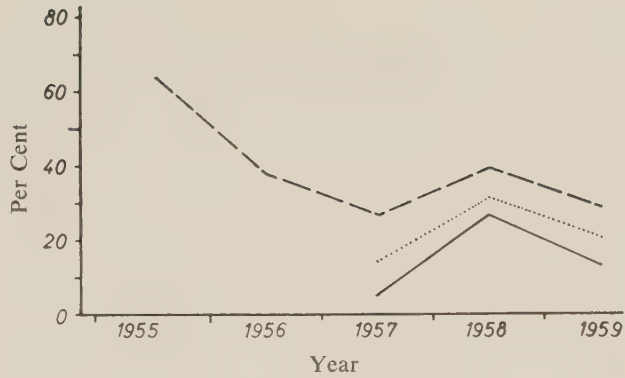
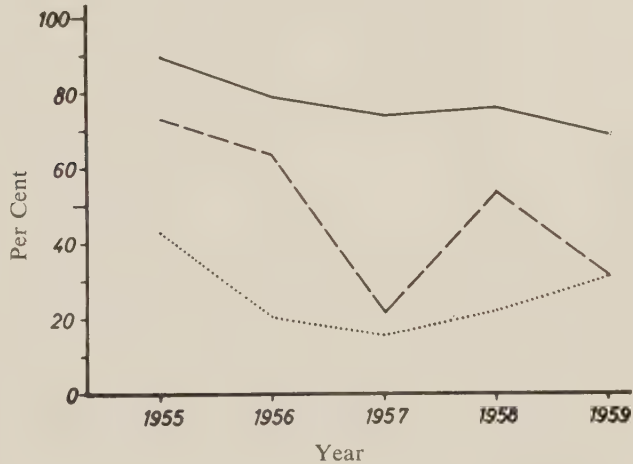


FIG. 2. Resistance of *Micrococcus pyogenes* against penicillin (———), chloramphenicol (— · — · —), and streptomycin (— — —).



the rules of general hygiene, as well as organizational and aseptic precautions which finally have led to a real reduction of bacteria (fig. 4).

It becomes apparent that the exclusive administration of one single antibiotic combination offers the possibility of having continuously controlled the development of resistance within a hospital as well as to maintain constantly an over-all view of the problem of resistance.^{2, 12, 13, 16}

As to the choice of the antibiotic for routine use, we are in favor of a combined preparation, since we hope thus to reduce the development of resistance at the

FIG. 3. The incidence of mastitis, 1949 through 1958.

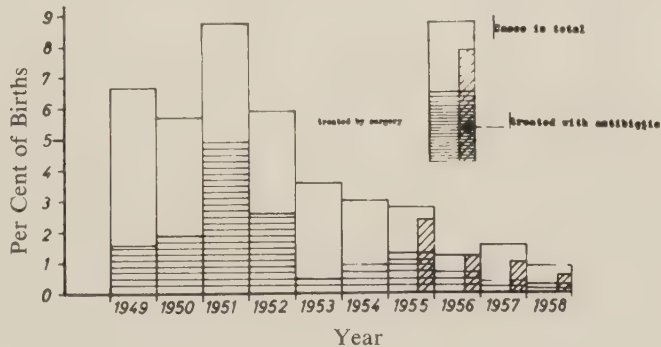


TABLE I

Incidence of Infections in Newborn Infants, 1955-1958

	1955	1956	1957	1958
Pyoderma	17	4	8	3
Paronychia	7	10	5	9
Mastitis	17	7	3	4
Omphalitis	13	3	8	3
Sublingual phlegmons	2	—	—	1
Pneumonia	1	—	2	1
Suppurative ocular secretion	1	—	2	1
Total	58	24	28	22
	in 1789	in 1800	in 1817	in 1788
Per cent	3.2	1.3	1.5	1.2

same time that a broader antibiotic spectrum is obtained.⁵ Although chloramphenicol was only needed in every fifteenth case, the development of resistance is in the same range as in the tetracycline-oleandomycin group. Thus it would seem that the combined preparation is more effective over a period of time, which was the experience of Maurer in the surgical field.¹⁴

Since we were able to demonstrate a retardation in the progress of development of resistance, the following must be included into the considerations: only 4 per cent of all patients were administered tetracycline-oleandomycin routinely in accordance with our strict limitations.

A further question still to be answered is how to explain the protracted decrease of the observed penicillin-streptomycin resistance curve. We are of the opinion that the extended and frequent use of these preparations by general practitioners must be made responsible for this development, since from these sources new resistant bacteria are introduced continuously.

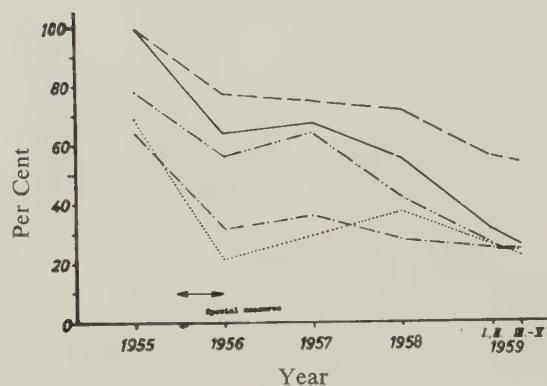


FIG. 4. Incidence of *Micrococcus pyogenes* in ward of newborn infants (—), in ward of women post-partum (— — —), in nasopharyngeal area of children (— · —), in papillae of women post-partum (— · —), and in nasopharyngeal area of nursing personnel (· · · · ·).

SUMMARY

Unfortunately, individual resistance tests cannot be carried out as quickly as necessary to control continuously the present problem. This, however, can only be brought about if there is a reduction of antibiotic consumption. Therefore, the use of a combined antibiotic preparation seems to be preferable because resistance does not develop so quickly.

In order to maintain the therapeutic value of antibiotics it is imperative to adhere strictly, to hygienic precautions, which are an effective help in the restriction of the selection process, since a real reduction of existent germs is thus effected.

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Three Years of Clinical Experience with Oleandomycin-Tetracycline Combination in the Treatment of Staphylococcal Infections

I. Clinical and Experimental Comparative Studies

JOSÉ LUIS MORADOR, SARA MORADOR, AND LOLA SALDAÑA TATE

*Blanco Acevedo's Medical Group for
Clinical and Laboratory Investigation, Hospital Pasteur, Montevideo, Uruguay*

Synergism and antagonism between antibiotics have been indicated some years ago. Since specific and fixed combinations of antibiotics have become available commercially, studies on their enhanced activity acquired practical significance. However, there is not yet a uniform criterion concerning the efficacy of antibiotics combinations in the treatment of staphylococcal infections.

Some authors have found clinical and experimental synergism of antibacterial effects, with certain combinations of antibiotics. In vitro studies conducted by English et al¹ demonstrated synergism between oleandomycin and tetracycline against resistant staphylococcal strains. In this study 8 out of 21 bacterial strains of antibiotic-resistant staphylococci were inhibited by lower concentrations of the mixture than required with the drugs employed alone. English also reported similar synergism in experiments conducted in mice—a high degree of protection for four days against a usually lethal infection. The protection was greater than could be expected through an additive effect of oleandomycin and tetracycline. Clinical uses of the antibacterial synergism of both drugs were described by Carter and Maley² (mainly in upper respiratory infections), by Winton and Chesrow,³ and by ourselves⁴⁻⁶ in two series of surgical patients and one series of anal and perineal infections.

This synergism was not found in vitro by Jones and Finland⁷ nor by Garrod.⁸ Elliott and Hall⁹ found that tetracycline was effective in enhancing the bacteriostatic activity of oleandomycin but less effective in increasing its bactericidal action. Johannessen and Ericsson¹⁰ have found only a weak and inconstant demonstrable synergism in vitro. Most recently, however, Mann¹¹ found that 76 to 80 per cent of test organisms (*Micrococcus pyogenes* var. *aureus*) were susceptible to a 1:2 combination of oleandomycin and tetracycline. Thus, he supports the work of English and others who previously demonstrated that an enhanced antibacterial effect was exhibited by the tetracycline-oleandomycin combination.

Our clinical observations showed us that an oleandomycin-tetracycline combination has considerably more activity than either of its two components when employed alone. We have also confirmed these findings in vitro, but not always and not constantly with all bacterial strains. Also, it was demonstrated in our laboratories¹¹ that there was a delay in the emergence of staphylococci resistant strains using the mixture. This paper is concerned only with the synergism of the antibiotic combination against coagulase-positive staphylococci and not with the problem of emergence of resistant strains. Our purpose is to compare: (1) clinical results obtained in the treatment of staphylococcal diseases with an oleandomycin-tetracycline combination; (2) similar conditions induced in experimental susceptible

TABLE I

*Surgical Patients with Staphylococcal Infections and Sources of Strains Isolated for Experimental Studies**

Clinical		Experimental, no. strains isolated
Diagnosis	No. patients	
<i>Skin and Soft-Tissue Infections</i>		
Benign		
Furunculosis	30	
Hidrosadenitis	18	
Breast abscess	13	7
Gluteal abscess	34	15
Deep felons	10	1
Serious		
Severe staphylococcal infection	9	
Extensive carbuncles in diabetic patients	5	3
Proliferative pyodermitis	1	
Total	120	26
<i>Bone Infections</i>		
Osteomyelitis	6	2†
<i>Infected Burns and Operative Wound Infections</i>		
Burns		
Outpatients, infection present on admission	5	1
Inpatients, staphylococci from hospital source	6	2
Wound infection	59	11
Total	70	14
<i>Severe Infected Conditions Caused by Staphylococci</i>		
Thoracic conditions		
Postoperative infections	2	1
Staphylococcal pneumonia	3	
Staphylococcal pulmonary infection	8	
Abdominal diseases		
Subphrenic abscess	2	2
Peritonitis	4	1
Superinfection	2	
Severe abdominal wound infections	19	10
Total	40	14

* A total of 236 patients with staphylococcal diseases were treated with oleandomycin-tetracycline combination; 56 strains of *Staphylococcus* isolated from clinical material were used in experimental study of synergism.

† Coagulase-negative, nontoxigenic strain.

animals (rabbits or guinea pigs) and prepared animals (irradiated or cortico-treated rats); and (3) determination in vitro of the sensitivity or resistance of the same strains of organisms isolated from the patients to the drug under study.

MATERIALS AND METHODS

In carrying out this work we used clinical and experimental material. The clinical material comprised the results of three years of experience with the oleandomycin-tetracycline combination in the treatment of staphylococcal diseases in surgical cases. Two hundred thirty-six* surgical cases with coagulase-positive staphylococcal infections treated with the antibiotic combination are distributed in four groups (table I).

* In this series are included 2 of 52 and 54 patients that were reported on before.^{4,5}

TABLE II

*Clinical Results Obtained in the Treatment of 236 Surgical Patients with Staphylococcal Infections with Tetracycline-Oleandomycin Combination**

Patient no.	Clinical condition	No. patients	Treatment		Clinical results
			Daily dose, mg.	Days (average)	
1-120	Skin and soft-tissue infection	120	750	3-12	100 per cent very good results
121-123	Chronic infection of bone	3	1000	20	3 good
124-125	Chronic osteomyelitis with acute exacerbation	2	1000	8	2 cured of acute condition
126	Brodie's abscess	1	1000	10	1 poor
127	Burn infected with drug-resistant organisms	1	1000	15	1 failure
128-137	Infected burn	10	750	12	10 very good
138-192	Operative wound infection	55	1000	15	19 very good and 36 good results
193-195	Operative wound infection	3	1000	15	3 failures
196	Operative wound infection	1	1000	5	1 interruption of treatment
197-208	Serious infected thoracic condition	12	1000	12	2 very good, 6 good, and 4 poor results
209	Bronchopulmonary infection; asthma	1	1000	5	1 failure
210-214	Abdominal infection	5	1000	12	2 very good, 3 good
215	Peritonitis	1	1000	10	1 failure
216-217	Superinfection	2	750	10	2 good
218-234	Serious abdominal wound infection	17	1000	12	9 very good, 3 good, and 5 poor
235		1	1000	10	1 failure
236		1	750	18	1 treatment interrupted

* There were 162 patients with very good results, 55 with good results, and 10 with only slight improvement or poor results; 7 patients failed to respond to treatment, and in 2 cases the therapy was interrupted due to intolerance.

Skin and Soft-Tissue Infections. One hundred twenty patients had skin and soft-tissue infections (furunculosis, breast and gluteal abscesses, hidrosadenitis, and deep felons); 15 of these cases were very severe. Serious infections included: 5 extensive carbuncles in diabetic patients, 1 proliferative pyodermitis, and 9 cases of soft-tissue infection in patients with a lowered resistance (diabetes, impaired circulation).

Bone Infections. Six patients had chronic bone infections due to *Staphylococcus aureus*, 3 with secondary contaminated sinuses.

Infected Burns and Operative Wound Infections. Eleven patients had second degree burns, 6 of them becoming infected during their hospital stay; the contamination was caused by resistant staphylococci from hospital sources. Five were outpatients and infection was present on admission. Staphylococcal post-operative infections were observed (mainly abdominal) in 59 patients.

Severe Infected Conditions Caused by Staphylococci. The series of 40 serious infections included 13 patients with thoracic conditions and 27 with abdominal

diseases. Among the thoracic infections there were 3 cases of pneumonia; 8 patients presented pulmonary infections with fever, cough, purulent sputum, and *Staph. aureus* positive on culture without evidence of pneumonia; 2 patients had pleural empyema after surgical drainage of traumatic hemothorax. Among the abdominal diseases there were: 2 subphrenic abscesses (1 corresponding to a secondary infection of primary clean surgical procedure); 4 peritonitis; 2 superinfections secondary to prolonged broad-spectrum antibiotic therapy; and 19 were patients with serious abdominal wound infections, 2 of these with abdominal parietal disruption and evisceration.

CLINICAL RESULTS

All these patients were treated with a combination of tetracycline and oleandomycin (2:1),* receiving by oral route from 750 to 1000 mg. daily in divided doses of 250 mg. each, over a treatment period ranging from 3 to 22 days. The results are given in table II.

Skin and Soft-Tissue Infections. In patients with skin and soft-tissue staphylococcal infections we obtained 100 per cent of very good results. In furunculosis there was a rapid subsidence of cellulitis and an arrest of the progressive necrotic condition. In some cases of gluteal and breast abscesses the suppurative process was satisfactorily controlled without surgical drainage. In the most serious infections occurring in patients with impaired resistance (mainly diabetics) we have had very good results in the control of the infectious condition.

Bone Infections. Three patients with fistulous chronic bone infections had mixed bacterial contamination. They were treated with the mixture with good results. Two with chronic osteomyelitis had acute exacerbations of their infective osseous process and were cured of the acute condition with tetracycline-oleandomycin in very few days. Another had a Brodie's abscess that yielded coagulase-negative *Staphylococcus* (strain 28); the result of therapy was only satisfactory.

Infected Burns and Operative Wound Infections. One patient with infected second degree burns had, on admission, a polybacterial infection with tetracycline- and oleandomycin-resistant organisms (*Proteus vulgaris* and *Pseudomonas aeruginosa*). This case failed to respond to treatment and was cured with chloramphenicol. Two patients with mixed (predominantly staphylococci), postoperative wound infections were contaminated by *Proteus* during treatment with tetracycline-oleandomycin. This organism was resistant to both drugs, and the patients were treated with chloramphenicol. One, a bad risk patient with impaired resistance (he had a far advanced cancer), developed a blood dyscrasia and died with granulocytopenia. A third patient had a staphylococcal infection after a pelvic surgical procedure that failed to respond to therapy. The organism, isolated from clinical material, was an oleandomycin-resistant *Staph. aureus* (strain 36) that was sensitive to erythromycin in vitro. For 14 days the patient received propionyl erythromycin,† in a dosage of 250 mg. of basic erythromycin every six hours, and recovered. The other patient was cured with the therapeutic treatment given.

* The trade name of Chas. Pfizer & Co. for tetracycline-oleandomycin (2:1) is Signemycin.

† The trade name of Eli Lilly & Co. for propionyl erythromycin is Ilosone.

TABLE III

Individual Responses in Staphylococcal Pulmonary Conditions

Patient no.	Clinical condition	Sputum smear and culture		Treatment		Results
		Before treatment	After treatment	Daily dose, mg.	Days	
199	Pneumonia	<i>Staph. aureus</i> , <i>K. pneumoniae</i> , pus cells	Few pus cells	750	8	Good
200	Pneumonia	<i>Staph. aureus</i> , hemolytic streptococci, <i>K. pneumoniae</i>	<i>K. pneumoniae</i> , moderate pus cells	1000	5	Good
201	Pneumonia	Purulent sputum	No pus cells	1000	6	Good
202	Bronchiectasis, acute infection	<i>Staph. aureus</i> , <i>K. pneumoniae</i>	No pus cells	750	16	Satisfactory, mucoid sputum
203	Bronchiectasis, acute infection	<i>Staph. aureus</i> , <i>K. pneumoniae</i>	Moderate pus cells	750	18	Slight improvement
204-208	Bronchopulmonary, postoperative acute condition	<i>Staphylococcus</i> predominating, mixed flora	No pus cells	Average 750	10 (av.)	Good
209	Bronchial asthma, chronic bronchitis with acute exacerbation	<i>Staph. aureus</i> , <i>K. pneumoniae</i> , <i>Str. viridans</i>	Moderate pus cells <i>Staph. aureus</i> , <i>Str. viridans</i>	750	5	Failure; treated with chlortetra- cycline for 8 days with improvement

Severe Infected Conditions. The results were very good in 2 patients with staphylococcal empyema produced after surgical drainage of a traumatic hemothorax. In respiratory diseases produced by staphylococci, the individual responses are presented in table III. The bacterial flora of the sputum was varied (hemolytic streptococci, *Klebsiella pneumoniae*, and *P. vulgaris*), with coagulase-positive strains of *Staphylococcus* predominating. Three patients had pneumonia and 8 bronchopulmonary infection; 2 of the latter had bronchiectasis with an acute condition superimposed. The therapeutic results were considered good and satisfactory in all but 1 patient. This was a man with bronchial asthma that had an acute exacerbation of a chronic bronchitis. Sputum smear and culture showed *Staph. aureus*, *K. pneumoniae*, and *Streptococcus viridans*. After treatment for five days with oleandomycin-tetracycline there was no improvement, and therapy was changed to chlortetracycline. After eight days the result of therapy was good. Ten patients showed clinical and roentgenological improvement, with decline in temperature, decrease in cough and amount of sputum, a change from a purulent to a mucoid sputum, and disappearance of roentgenological signs of the acute pulmonary condition.

Patients with abdominal diseases had the following evolutive course: 2 subphrenic abscesses, after clean laparotomy, were cured with the treatment; 2 cases of superinfection after prolonged broad-spectrum antibiotic therapy were cured with the mixture under study. In 4 cases of peritonitis with abdominal drainage and mixed polybacterial wound infection, the results were good in 3 and poor in 1 patient, who had a wound contaminated with *Escherichia coli*, *Proteus*, and *Ps.*

aeruginosa. Staphylococci were also present in the wound but in few days were substituted by the predominating *Proteus*, resistant to all antibiotics.

Serious staphylococcal postoperative infections, after adequate surgical treatment, were cured with oleandomycin-tetracycline in all but 1 case. This was a 64 year old man with a severe necrotic suppurative infection produced by oleandomycin-resistant *Staphylococcus*. Treatment with the combination of antibiotics failed, and only poor results were obtained with an interrupted therapy with chloramphenicol.

Summary. In 236 patients with varied staphylococcal infections, the clinical results obtained with oleandomycin-tetracycline were as follows: in 162 patients, very good; in 55 patients, good; only satisfactory or poor results were obtained in 10 patients. In 2 cases it was necessary to discontinue therapy. One was due to the appearance of diarrhea and the other to moniliasis. Seven patients failed to respond to treatment, and it was necessary to change the medication with varied results (table IV). Four patients were treated with chloramphenicol, 1 with erythromycin, and 1 with chlortetracycline. There were two negative results, one due to organisms resistant to all antibiotics (*P. vulgaris* and *Ps. aeruginosa*). Another patient died with granulocytopenia.

EXPERIMENTAL MATERIAL

The experimental material used in this work comprised 56 staphylococcal strains isolated from varied sources during the routine work of the clinical bacteriological laboratory; the sources were mainly pus from soft-tissue infection, carbuncles, osteomyelitis, burns, wound infections, and thoracic and abdominal infections, such as pleural empyema, subphrenic abscess, and peritonitis. These strains were tested for sensitivity to tetracycline and oleandomycin, separately and in combination,

TABLE IV

Observation of 7 Patients Who Failed to Respond to Therapy with Tetracycline-Oleandomycin

Patient no.	Clinical condition	Bacteriological finding	Replacement antibiotic	Result
127	Burn, second degree	<i>Proteus</i> , <i>Ps. aeruginosa</i> , <i>Staph. aureus</i>	Chloramphenicol	Very good
193	Infected operative wound, far-advanced cancer	Secondary infection with <i>Proteus</i>	Chloramphenicol	Died; granulocytopenia
194	Operative wound	Secondary infection with <i>Proteus</i>	Chloramphenicol	Good
195	Operative wound	Oleandomycin-resistant <i>Staphylococcus</i> (strain 36)	Erythromycin	Very good
209	Respiratory infection, asthma	<i>Str. viridans</i> , <i>K. pneumoniae</i>	Chlortetracycline	Good
215	Peritonitis	Mixed flora; <i>E. coli</i> , <i>Proteus</i> , <i>Ps. aeruginosa</i> (strain 46)	— *	Negative
235	Serious wound infection	Oleandomycin-resistant <i>Staphylococcus</i>	Chloramphenicol	Poor

* Infection resistant to all antibiotics.

TABLE V
In Vitro Evidence of Synergism

Number of cultures	Clinical condition	Minimal inhibitory concentration, $\mu\text{g./ml.}$				Clinical results
		Antibiotics alone		Antibiotic combination		
		Oleando- mycin	Tetra- cycline	Oleando- mycin	Tetra- cycline	
8	Breast abscess*	0.25	15.6	0.062	3.9	Marked evidence of synergism
		2.0	62.5	0.5	15.6	
6	Gluteal abscess*	0.5	3.9	0.062	0.5	Marked evidence of synergism
		2.0	125.	0.5	31.2	
1	Deep felon	2.0	31.2	0.25	3.9	Evidence of synergism
2	Carbuncle	0.25	0.5	0.062	0.125	Synergism
		0.5	15.6	0.125	3.9	
1	Osteomyelitis	1.0	7.8	0.062	0.5	
5	Operative wound* infection	0.25	0.25	0.062	0.125	
		2.0	31.2	0.25	3.9	Marked evidence of synergism
2	Subphrenic abscess	1.0	7.8	0.062	0.5	
		0.5	15.6	0.125	3.9	Evidence of synergism
	Pleural empyema	2.0	62.5	0.5	7.8	
6	Serious post-operative abdominal infection*	0.25	7.8	0.062	2.0	
		2.00	125.	0.5	62.5	

* Only lowest and highest values in the series are given.

in the proportion obtainable commercially (67 per cent of tetracycline and 33 per cent of oleandomycin).

The biological characteristics of the organisms studied were determined by the presence of pigment and the enzymatic activities (coagulase, fibrinolysin, and hemolysin production). Their pathogenicity was observed experimentally in animals. Methods employed in the determination of the biological characteristics are described elsewhere.¹²

The bacterial sensitivities to oleandomycin and tetracycline were determined by tube dilution and agar diffusion methods. In the tube dilution test method we used oleandomycin phosphate and tetracycline hydrochloride dissolved in distilled water to give 1000 $\mu\text{g./ml.}$ This stock solution was filtered and stored frozen no longer than one month. Every organism was tested for susceptibility to oleandomycin and tetracycline alone, using the twofold serial dilution tube technique, as described by Welch et al.¹³

With final volume of 1.0 ml. [0.5 ml. of serially diluted antibiotic in broth and 0.5 ml. of the dilution culture (1:1000)], we determined after 24 hours of incubation at 37 C. the minimal inhibitory concentration. On testing oleandomycin, we also used the determination of the maximum number of cells inhibited by an arbitrarily determined concentration of drug.⁵ The bactericidal end point for each antibiotic preparation was elicited by subculture of each broth tube to an agar plate.

The antibacterial effect of the combined drugs was evaluated comparing the minimum inhibitory concentration of the two antibiotics alone with the concentration of each needed to obtain inhibitory effect when used in combination.

TABLE VI

Bactericidal Effect of Oleandomycin-Tetracycline in Susceptible Strains of Staphylococci, as Determined by Replica Plate Method

Strain no.	Tetracycline	Oleandomycin	Mixture	Effects seen in primary plate
5	12*	18	5†	Synergism
9	8	20	8	Synergism
15	7	8	7	Synergism
28	14	24	5†	No synergism
44	5	9	2†	Synergism

* Number of bacterial colonies seen on the replica plate corresponding to the zone of inhibition in the primary plate.

† Enhanced bactericidal activity of tetracycline.

We have also used the agar diffusion method for routine testing of bacterial sensitivity to combinations of antibiotics. The sensitivity of the organism to the mixture was determined with the aid of a paper disc method, according to Johannessen and Ericsson,¹⁰ or employing the paper strip method. On employing the agar diffusion method, we used replica plating in routine work (according to the technique described by Lederberg and Lederberg¹⁴ for the selection of mutants) to determine any bactericidal action of the individual antibiotics or an increased bactericidal effect of combinations of the drugs. Synergism of antibacterial effects was recorded according to definition* by Price et al.¹⁵

EXPERIMENTAL RESULTS

Oleandomycin-tetracycline acted with synergism against 31 of the 56 cultures (57.1 per cent). No antagonism between the drugs was observed. For each of the susceptible strains affected synergistically, table V shows the minimum inhibitory concentrations of oleandomycin and tetracycline alone and in combination.

Replica plates were prepared from five of the primary plates with susceptible strains. The synergism observed with the agar diffusion method was reproduced on the replica plates. Oleandomycin seemed to increase the bactericidal action of tetracycline. In evaluating this bactericidal effect, we have used the classification of Mantén.¹⁷ The inhibition zone of the primary plate, when transferred to a replica plate, showed bactericidal potency ascribed to the combination of antibiotics, since on the latter no more than five bacterial colonies developed, and indicated only partial bactericidal action when more than 5 and less than 25 colonies, developed. Table VI shows these results.

All the cultures listed in table V were sensitive to from 0.25 to 2.0 µg./ml. of oleandomycin, except two that had minimum inhibitory concentration values of 3.9 µg./ml.; 23 of the cultures were sensitive to less than 15.6 µg./ml. of tetracycline, while nine were resistant to from 15.6 to 125 µg./ml. Of the remainder, seven strains were oleandomycin resistant. All these strains but one (strain 36) were also resistant

* The last year we adopted partially the definition given in a paper by Garrett,¹⁶ mainly for the concept of equivalence and additivity. The figures so obtained are not recorded here so as to give uniform and easily comparable records throughout the three years.

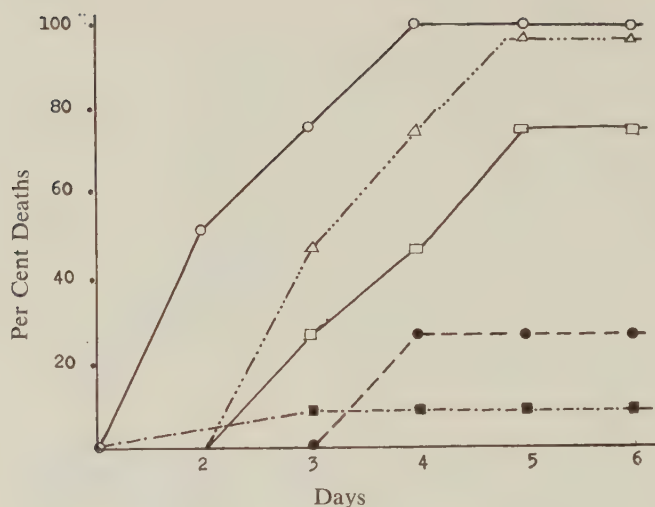


FIG. 1. Synergism measured determining the four days' protection against a usually fatal inoculae of *Staphylococcus aureus*. Each point equals 5 animals. o—o, control; ■-■, second group (15 strains); ●-●, third group (5 strains); □—□, fourth group (4 strains); △-...-△, fifth group (1 strain).

to erythromycin. In all cases the minimum inhibitory concentration was determined with recently isolated strains.

In 24 instances the minimum inhibitory concentration of the mixture was generally the same as that of the more active of its two components or one or two tubes higher. Nevertheless, the clinical results obtained in the treatment of the patients infected with these organisms implied marked evidence of simple synergism. It seems that the mechanism of synergism *in vitro* does not have an exact relation to clinical application, and oleandomycin and tetracycline may display synergism *in vivo* through a more complicated and still unknown mechanism. We have not studied here the cross resistance between oleandomycin and erythromycin. It is fully confirmed that a strain resistant to erythromycin may or may not be resistant to oleandomycin. The contrary is not common, but we have found one strain (no. 36) of these 24 strains that was erythromycin sensitive (0.5 $\mu\text{g./ml.}$) and oleandomycin resistant (15.6 $\mu\text{g./ml.}$). (See table IV.)

IN VIVO EXPERIMENTS

The experiments were carried out in susceptible animals (rabbits or guinea pigs) and in prepared animals (irradiated and cortico-treated rats). Each animal received intravenously or intraperitoneally 0.1 or 0.5 ml. of a staphylococcal culture of 24 hours at 37 C. All the inocula consisted of organisms freshly isolated from clinical material. The experiments were divided in five groups, as follows: (1) inoculum: pathogenic, coagulase-positive staphylococci; treatment: none (control group); (2) inoculum: oleandomycin-sensitive *Staph. aureus* (strains synergistically affected by oleandomycin-tetracycline combination *in vitro* and in disease; treatment: antibiotic mixture and each drug alone from the first day; (3) inoculum: oleandomycin- and/or tetracycline-resistant organisms (strains synergistically affected clinically, no synergism *in vitro*); treatment: tetracycline-oleandomycin combination administered from the first day and both drugs used alone; (4) inoculum: strains obtained from patients who showed poor clinical response to treatment (affected by additivity *in vitro*, no synergism); treatment: mixture from the first day and oleando-

TABLE VII

*Correlation of Bacteriological Data with Clinical Responses to Treatment
and Animal Experiment Results*

Group no.	Clinical response to treatment	In vitro determination of synergism*	No. strains employed in vivo	Experimental in vivo results
2	Clinical evidence of synergism,† 100 per cent	Synergism in 57.1 per cent of strains studied§	15	Synergism, 93.4 per cent
3	Clinical evidence of synergism, 100 per cent	No synergism in 42.9 per cent of strains studied	5	Synergism, 80 per cent
4	No synergism, only additivity,‡ 100 per cent	No synergism, 100 per cent	4	No synergism, 80 per cent
5	No synergism, 100 per cent	No synergism, 100 per cent	1 (strain 36)	No synergism, 100 per cent

* Synergism was determined by twofold serial dilution tube method.

† Clinical evidence of synergistic action was considered when the clinical response to treatment was very good.

‡ Additivity corresponds to a poor clinical response.

§ Percentage obtained from 56 strains.

|| Synergism measured in four days protection against usually fatal inoculum of *Staphylococcus aureus*.

mycin and tetracycline alone; (5) inoculum: oleandomycin-resistant *Staph. aureus* 36 (strain unaffected by the mixture of antibiotics either clinically or in vitro).

Synergism was measured in each of the last four groups, determining the degree of protection for four days against a usually fatal inoculum, compared with the control group of animals and with the series receiving each drug alone.

IN VIVO RESULTS

The in vivo results are summarized in figure 1. In the first group, all the animals that were inoculated died. In the second group, antibiotic protection was obtained in almost 100 per cent of the inoculated animals. In the third group, five strains were inoculated, and we found synergism against four. In the fourth group no synergistic protection was found in three of four strains studied. The affected strain was no. 28 (from a case of osteomyelitis), and the result has no value because the organisms isolated from osteomyelitis cases do not kill as many animals as do organisms from other sources. In the fifth group (oleandomycin-resistant strain 36), the antibiotic mixture failed to protect the animals. In all the experiments, technique and doses were the same as those used before.¹⁸ The protection afforded was greater than could be expected with addition of the protective effects of oleandomycin and tetracycline employed alone.

DISCUSSION

Since it is not fully accepted that tetracycline-oleandomycin combination has synergistic action against *Staph. aureus*, it was considered of interest to study effects experimentally in vitro and in vivo and to compare them with the clinical results obtained in the treatment of surgical patients with staphylococcal diseases

(table VII). The present studies confirm the fact that the mixture is more active than either component alone.

Of 236 patients treated with the antibiotic combination, in only 7 did the treatment fail and in 2 instances have to be interrupted; 10 patients responded poorly to treatment; synergism was found in 91.7 per cent of the patients treated.

The potentiation, measured by minimum inhibitory concentration (twofold serial dilution tube), was synergistic in 57.1 per cent of the strains studied.

The synergistic activity of the mixture against experimental infections produced by *Staph. aureus* confirms the clinical results and also the findings in vitro when both are coincident (i.e., clinical and in vitro synergism found in the second group, or clinical and in vitro failure of synergism as seen in the fifth group). When the combination of oleandomycin and tetracycline is used against organisms that clinically were synergistically affected by the drugs but not in vitro, the mixture achieves a percentage of protection greater than that which could be anticipated from the minimum inhibitory concentration obtained in vitro. On the other hand, in experimental infections induced by strains of organisms found resistant in vitro, the protection afforded was—as clinical results anticipated—the same as that corresponding to the sum of the component activities (additive protection).

A comparison of the studies carried out with oleandomycin-tetracycline combination in the treatment of staphylococcal diseases, in the in vitro evaluation of synergism, and in the protection against experimentally induced infection shows us that: (1) this antibiotic combination is considerably more active than either component alone; and (2) evidence of synergism was found in 91.7 per cent of the patients treated and confirmed in experiments.

SUMMARY

1. A series of 236 surgical patients with staphylococcal infections were treated with oleandomycin-tetracycline combination.
2. Synergism was evident in 91.7 per cent of the treated patients.
3. The potentiation measured with twofold serial dilution tube technique was synergistic in 57.1 per cent of the strains studied.
4. Oleandomycin-resistant staphylococci increased in three years in our hospital from 0 to 14.3 per cent.
5. The synergistic activity of oleandomycin-tetracycline combination in experimental infections produced by *Staph. aureus* in rabbits or guinea pigs afforded a protection greater than could be expected through the addition of the protective effects of each drug.

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A Nine Year Study of Coagulase-Positive Staphylococci in a Burn Unit: Incidence, Resistance Pattern, Personnel Carriers, and Phage Typing*

B. W. HAYNES, JR., VIRGINIA JONES, AND COUNT D. GIBSON, JR.

Department of Surgery, Medical College of Virginia, Richmond, Va., and Department of Preventive Medicine, Tufts University School of Medicine, Boston, Mass.

The Burn Unit of the Medical College of Virginia Hospital is composed of two wards containing 26 beds that are used for the treatment of burn patients exclusively. Approximately 150 patients are admitted yearly, and the nursing staff is made up chiefly of personnel who have worked in the area exclusively for several years. Dressings are changed in a special air-conditioned dressing room to minimize cross contamination of bacteria between patients. A special bacteriology laboratory has been in operation during the time of this study. The major function of this laboratory has been the performance of culture and sensitivity studies on burn wounds; these data form the basis for this report.

MATERIALS AND METHODS

Each patient admitted to the unit ordinarily had the burn wound cultured immediately and at each dressing change. Initial dressings were usually changed in 7 to 10 days; subsequent dressings at 5 to 7 day intervals. Surgical débridement and wound cleansing were emphasized at each dressing change, leading to the development of a clean surgical wound at the earliest possible date (usually three to four weeks), which was followed by prompt split skin grafting. Cultures so obtained were planted promptly to assure growth, and identification of organisms was carried out by standard techniques. Sensitivity testing was performed by the disc method until mid-1954; since then, by the pour plate method. The organism was tested against two quantities of antibiotic to determine sensitivity: bacitracin, 2 units and 20 units; chloramphenicol, 10 and 30 µg.; erythromycin, 10 and 30 µg.; neomycin sulfate, 15 and 30 µg.; penicillin G potassium, 1 and 10 units; polymyxin B sulfate, 5 and 20 µg.; dihydrostreptomycin, 15 and 30 µg.; and tetracycline hydrochloride, 3 and 10 µg. Organism sensitivity or resistance was defined as follows: inhibition of growth in high antibiotic concentration only—moderately sensitive; inhibition of growth in high and low antibiotic concentration—sensitive; no inhibition of growth in either concentration—resistant.

In general, antibiotics were administered in standard dosages.

RESULTS AND DISCUSSION

Incidence. Data on total cultures, number of patients cultured, and number of staphylococci found are summarized in table I. It will be observed that the per

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TABLE I
Burn Wound Cultures (1951-1959)

Year	Cultures			Patients		
	Total no. cultures	Total no. staphylococci	%	No. patients cultured	No. patients with staphylococci	%
1951	287	75	26	67	20	30
1952	785	301	38	246	75	30
1953	344	196	57	120	68	57
1954	335	176	52	104	73	70
1955	473	243	51	111	77	69
1956	341	183	54	90	62	69
1957	375	243	65	78	63	81
1958	525	357	68	125	99	79
1959 (Jan.- June)	205	163	80	55	49	89

cent of total cultures positive for *Staphylococcus aureus* rose from 26 per cent in 1951 to 80 per cent in 1959. There seems to be a plateau in incidence during the years 1953-1956, which is preceded and followed by a sharp rise. At the same time, the number of patients demonstrating positive staphylococci increased from 30 per cent of the patients cultured in 1951 to 89 per cent in 1959, with a similar suggestion of a plateau effect during the year 1954-1956. These data are graphically presented in figure 1.

There are, therefore, two points of interest in these data. First, the distressing

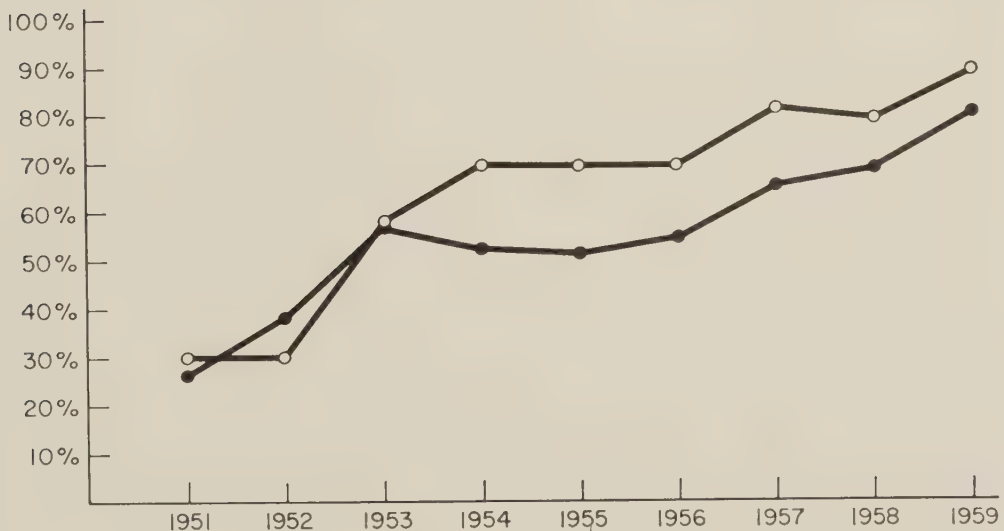


FIG. 1. The incidence of coagulase-positive *Staph. aureus* in a burn unit. A rising incidence of staphylococci in burn patients and in burn wound cultures is demonstrated. The significance of the plateau occurring during the years 1953 to 1956 is commented upon in the text. ○—○, per cent of patients with staphylococci; ●—●, per cent of total cultures with staphylococci.

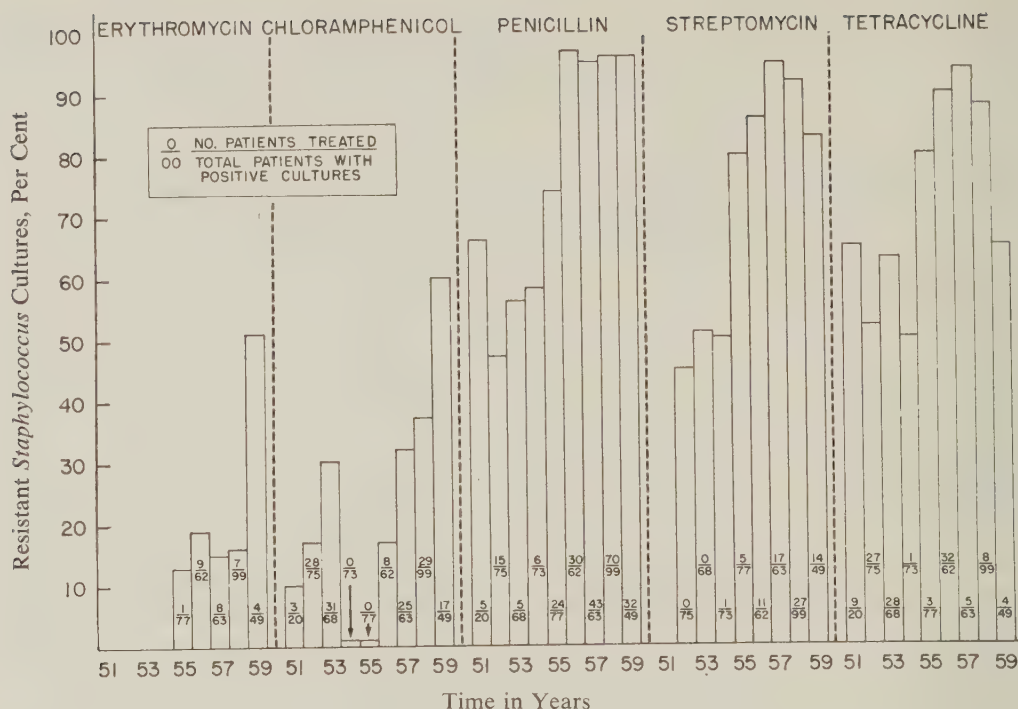


FIG. 2. The resistance pattern of coagulase-positive *Staph. aureus* in burn wounds with reference to antibiotic usage. The pattern of staphylococcal resistance to a group of antibiotics over a nine year period shows a correlation between resistance and antibiotic usage.

tendency for the *Staphylococcus* to increase both in the percentage of cultures and in the percentage of patients is evident. Second, the plateau noted in the curves of incidence during the years 1953–1956 seems to be a real interpretation of the data. It is interesting that the years 1954 and 1955 were marked by virtually a complete withdrawal from use in the unit of chloramphenicol and tetracycline. Also, erythromycin and streptomycin were used infrequently, and the principal antibiotic given was penicillin (see subsequent section on resistance pattern). In subsequent years, all agents were used to a greater extent, especially chloramphenicol. The steep portions of the curve indicating increasing incidence of staphylococcal infection correspond to the time when intensive broad-spectrum antibiotic therapy was employed. The plateau of the curve indicating no increase in incidence of infection corresponds to the phase of treatment when the broad-spectrum antibiotics were withheld. These data suggest that these potent broad-spectrum antibiotics are producing a strain of resistant staphylococci, which are colonizing the wounds in increasing frequency. Data on the resistance pattern of staphylococci reported below support this interpretation. On this basis, avoidance of prophylactic antibiotic treatment and the institution of a rotational system of antibiotic therapy seems mandatory to stem the rising tide of resistant staphylococci.

Resistance Pattern. The frequency of staphylococcal resistance to the common antibiotics has been documented by year and is presented in figure 2. For better evaluation of these data, the antibiotic usage factor has been presented for each antibiotic each year. The numerator in each bar represents the number of patients

treated during the year with that antibiotic; the denominator represents the total number of patients with positive staphylococcal cultures for the year. Some of these data have been reported previously but are included here to demonstrate trends over a longer period.¹

Erythromycin was first used in 1955, at which time the observed frequency of staphylococcal resistance was 13 per cent. The pattern of resistance remained about the same until 1959, when 51 per cent of the cultures of 49 patients with staphylococci demonstrated resistant organisms. The explanation for this change is not apparent. No significant amount of oleandomycin was used that might have produced cross resistance to erythromycin. The quantity of erythromycin used would not suggest an explanation (fig. 2).

The data on chloramphenicol are of interest chiefly because of the fall in numbers of organisms demonstrating resistance associated with discontinuance of the drug during the years 1954–1955. The abrupt decrease in resistant strains to very low levels during 1954–1955 requires explanation. Careful review of the data show that very little chloramphenicol was used during the last six months of 1953 or during the first six months of 1956. The decrease in resistant strains began during the latter half of 1953 and continued during the first half of 1956. Resistant strains observed during the year 1956 (fig. 2) occurred mainly during the last six months of the year. The possible effect withdrawal of chloramphenicol may have had on incidence of staphylococci in the wounds has been commented on. The increasing frequency of resistance with increasing antibiotic usage both before and after the period of drug withdrawal suggests a high degree of correlation between usage and the development of resistant strains.

The frequency of staphylococcal resistance to penicillin was high in 1951 and approached 100 per cent in 1956, where it has remained to date. It should be pointed out that penicillin was given primarily because of the continuing susceptibility of the hemolytic *Streptococcus* to it. Furthermore, it is our policy to treat all severely burned patients on admission to the hospital with penicillin and streptomycin prophylactically. This pattern of resistance to penicillin suggests a correlation between penicillin usage and penicillin resistance similar to that of chloramphenicol. It also suggests that the use of penicillin prophylactically be re-evaluated in the light of these resistance data and its use discontinued. The burn wound commonly contains a mixed flora of organisms including *Staph. aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Aerobacter aerogenes*, and hemolytic *Streptococcus*. Of this group, only the *Streptococcus* retains its sensitivity to penicillin, since the incidence of penicillin-resistant staphylococcal strains now approaches 100 per cent. In weighing the hazards of development of streptococcal infection against the hazards of production of penicillin-resistant staphylococcal strains, the former is the lesser of two evils. Should a streptococcal invasive infection occur after discontinuing the use of penicillin prophylactically, it could certainly be treated more effectively than a resistant staphylococcal infection developing under penicillin therapy. Since staphylococci develop resistance patterns to penicillin and other broad-spectrum antibiotics similarly, it is believed that prophylactic therapy with these agents is contraindicated.

The frequency of staphylococcal resistance to streptomycin is similar to that with penicillin except that the former is not quite so pronounced. Again, develop-

TABLE II
Incidence of Coagulase-Positive Staph. aureus in Burn Ward Personnel Cultures

	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1956												
No. cultures possible	37	37	37	37	37	37	35	34	34	34	34	34
No. cultures taken	—	37	35	35	36	36	35	32	34	34	34	33
No. finger positive	—	4	2	2	2	6	1	0	0	5	0	6
No. throat positive	—	0	0	0	0	0	0	0	1	0	1	0
No. nose positive	—	7	7	7	10	9	6	6	8	9	10	11
1957												
No. cultures possible	33	33	33	33	33	33	33	33	34	34	34	34
No. cultures taken	—	33	—	22	32	33	—	32	34	32	34	27
No. fingers positive	—	3	—	0	3	0	—	5	1	5	3	1
No. throat positive	—	0	—	0	0	0	—	0	0	0	0	0
No. nose positive	—	9	—	6	13	9	—	11	15	7	7	8
1958												
No. cultures possible	35	35	35	35	35	35	35	32	32	32	32	—
No. cultures taken	32	35	—	34	34	33	—	30	27	29	30	—
No. finger positive	6	7	—	4	8	3	—	6	1	3	2	—
No. throat positive	0	0	—	0	1	2	—	1	0	0	0	—
No. nose positive	9	11	—	9	6	11	—	12	9	10	9	—
1959												
No. cultures possible	33	33	33	33	33	33						
No. cultures taken	33	28	29	29	27	32						
No. finger positive	3	3	4	4	3	9						
No. throat positive	0	1	0	0	0	0						
No. nose positive	8	9	8	10	9	8						

ment of resistance would seem to be associated with usage, since approximately twice as many patients received penicillin as received streptomycin.

The frequency of resistance to tetracycline began at a high level and increased to almost 95 per cent of staphylococci cultured in 1957. In the past three years, because of virtually complete resistance to this agent, usage has decreased. This may account for the trend toward the increasing frequency of sensitivity of staphylococci to this agent. However, during the years 1954 and 1955, very little tetracycline was used; yet a striking increase in frequency of resistance was observed. There seems to be no obvious explanation for this change.

These data on staphylococcal resistance patterns support the concept that the continued use of penicillin and other potent antibiotics is instrumental in producing a staphylococcal population of increasingly resistant strains that are colonizing the burn wounds in increasing frequency. On the basis of past experience, adding a new broad-spectrum antibiotic to the treatment regimen can be expected to produce a similar resistance pattern. The notable exceptions to this statement (bacitracin, neomycin, and polymyxin) are unfortunately significantly more toxic than the group shown in figure 2 and should be reserved for the more severe staphylococcal infections. For these reasons, a reasonable solution to the problem of the antibiotic therapy of burn wound infection would be a strict rotational program of antibiotic therapy controlled by culture and sensitivity data. Many problems would be raised in carrying out such a program. However, until such time as antibiotic therapy can become as specific for the offending organism as bacteriophage

typing is for the identification of various staphylococcal strains, abolishment of indiscriminate antibiotic use is the only sensible approach to the problem.

Although not included in figure 2, data on staphylococcal resistance to bacitracin, neomycin, and polymyxin B during the same period have been collected. In general, it can be said that no significant resistance to these agents has been observed. Usage has been limited chiefly to a study of the local application of these agents on one or more occasions to the wounds of 106 patients from 1955–1958. The local use of these agents has produced no significant reduction in positive *Staphylococcus* cultures.

Personnel Carriers. To assess the importance of ward personnel as carriers of staphylococci from patient to patient, nose, throat, and finger cultures were made monthly beginning January, 1956, and continuing through June, 1959. The turnover of nursing and housekeeping personnel was minimal during this period; the main changes occurred in the residents and in hourly nursing personnel used to supplement the regular staff. Since this program was instituted to gain information on the existing situation, local or general treatment of personnel carriers has not been attempted. These data are presented in table II.

It will be observed that a high proportion of the ward personnel were cultured from month to month, the usual sample being in excess of 90 per cent of the personnel.

The most surprising finding is the incidence of the carrier rate for staphylococci in the nose. The rate has varied from 17 per cent to 44 per cent of those persons cultured. This is of interest; because the reported incidence of hospital personnel carriers of staphylococci is much higher—99.2 per cent in 1953, 75.3 per cent in 1955, and 52.3 per cent in 1956.² Though these figures contain data on patient carriers as well as on personnel carriers, it is concluded that the personnel carrier rate is much higher than that reported in our study. One might expect a high nasal carrier rate in persons working in a burn ward, where air-borne staphylococci are common and chances for finger contamination are frequent. The reason for the difference is not clear but may be related primarily to personnel resistance factors rather than to the environment.

A second point of interest is the relative consistency of the carrier rate in personnel from winter to summer. Since burns are a seasonal injury, there is a large fluctuation in the patient load from summer to winter. During December, January, and February, patient census often reaches 35 to 40; while in July and August it may fall to 12 to 15. If environmental load of staphylococci were primarily responsible for the nasal carrier rate in personnel, one would expect a seasonal shift. As seen in table II, such was not the case. Furthermore, less than half the number of persons showing positive nasal cultures were chronic carriers; the rest of the carrier group included many whose nasal cultures were positive for a few months and then became negative. These observations re-emphasize the point that factors operating in the individual may be more important with regard to the nasal carrier state than the environmental staphylococcal load.

It is apparent that the nose is colonized more frequently by staphylococci than the throat, and the number of positive finger cultures points out the importance of cleanliness of personnel. The significance of contaminated fingers as a source of cross contamination between patients is pointed out later.

TABLE III

Data on Phage Typing of 212 Staphylococci from Burn Wounds

Type	Number (%)	% of those typable
Nontypable	77 (36)	
53/75	38 (18)	28
75/77	54 (25)	40
7	10 (8)	7
80/81	10 (8)	7
53	5 (2)	4
77	10 (8)	7
54/75	3 (1)	2
Va ₄	3 (1)	2
75	1 (0.5)	0.7
54	1 (0.5)	0.7

Bacteriophage Typing. Since the summer of 1958, phage typing has been carried out on 212 staphylococci isolated from burn wounds. These data are presented in table III.

The majority of the typable staphylococci fell into 53/75 and 75/77 types (68 per cent). These types are a part of group III, which contains the most common types seen in surgical patients. Virtually all of these staphylococci were penicillin resistant.

Of 19 coagulase-positive staphylococci isolated from nasal cultures of burn ward personnel, no isolate was of the 53/75 or 75/77 pattern found on 43 per cent of the wounds (see table IV). One type 80/81 was found, a type which was seen in 8 per cent of the wounds. However, of eight cultures of staphylococci from the fingers of ward personnel, two were type 77 and one was type 53. In our burn unit, it would seem that contaminated fingers may serve as a means of cross-contamination between patients but that nasal carriers do not. A similar conclusion was reached by Caswell et al³ in a group of hospital infections.

TABLE IV

Phage Typing of Nasal Carriers of Staph. aureus (19 Cultures)

No. of cultures	Phage type
6	Nontypable
2	3 C, 3 A
2	6
1	55
1	80/81
2	52 A, 79, 77, 55
2	7
1	52, 80, 81
1	3 C, 7
1	3 A

SUMMARY

A nine year study of coagulase-positive *Staph. aureus* in burn wounds may be summarized as follows:

1. *Incidence.* A rising incidence of staphylococci both in percentage of cultures and in numbers of patients is demonstrated. This incidence is now approaching 100 per cent. The evidence suggests that this is a result of the use of potent antibiotic agents. On this basis, avoidance of prophylactic antibiotic treatment and institution of a rotational system of antibiotic treatment seems mandatory.

2. *Resistance Pattern.* The data presented suggest a correlation between antibiotic usage and development of resistance, especially with regard to chloramphenicol, penicillin, streptomycin, and tetracycline. The need for a rotational system of therapy is further emphasized by these data. The value of prophylactic penicillin therapy is questioned, since it is believed that the hazard of development of streptococcal infection is less risk to the patient than fostering the growth of resistant staphylococcal strains and the development of staphylococcal infection.

3. *Personnel Carriers and Phage Typing.* Nasal cultures of burn ward personnel have shown them to be harboring phage types of staphylococci rarely found in the burn wounds. Nasal carriers, therefore, did not constitute a significant source of wound contamination in this study. It is believed that the cause of the nasal carrier state is more closely related to resistance factors in the individual than to environmental staphylococcal load. Personnel with contaminated fingers are believed to be a more important source of cross contamination than are the nasal carriers.

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Bacteriological Study of Clean Surgical Incisions and the Personnel of an 800 Bed Hospital

WILLIAM W. WRIGHT, ELIZABETH J. OSWALD, ROBERT J. REEDY,
DOROTHY W. POCURULL, AND HOWARD I. WEINSTEIN

*Food and Drug Administration, U. S. Department of Health, Education, and Welfare,
and Washington Hospital Center, Washington, D. C.*

Infections involving antibiotic-resistant microorganisms have become a serious problem in some hospitals. Outbreaks of serious infections caused particularly by antibiotic-resistant coagulase-positive strains of *Staphylococcus aureus* have been reported from a number of hospitals as originating in nurseries and in maternity, medical, and surgical wards. Factors implicated in the initiation of such epidemics are many. The antibiotic-resistant strains become prevalent when the concentrated use of certain antibiotics eradicates susceptible strains. Forming a vicious circle, the resistant strains appear in infections and may predominate in the nasal flora of persons known formerly to be carriers of otherwise harmless strains. The carriers include hospital personnel (nurses, physicians, orderlies) and patients. The pathogenic strain spreads in innumerable ways, including direct contact, circulation of contaminated air, dust, and aerosols from breathing, coughing, sneezing, moving about, and cleaning, as well as by contact with contaminated fomites such as bedding, equipment, and furniture.

While a number of hospitals in this country have been unfortunate enough to have epidemics descend on them, the great majority have escaped. Nevertheless, the possibility that an epidemic could develop has made it imperative that certain preventive measures be delineated and observed. Among the recommendations for controlling and preventing staphylococcal infections in hospitals has been the establishment in each hospital of a committee on infections to review all infections, to promote procedures to prevent the transmission of infections, and to detect and abort potentially dangerous situations.¹

This study was undertaken at the Washington Hospital Center when it had been in operation for about 18 months. Based on the records of the Committee on Hospital Infections there was, ostensibly, no infection problem of any proportion. For this reason it was desirable to study surgical patients, in whom hospital infection was most likely to develop. Since we were interested in the transmission of organisms into otherwise sterile incisions, the incisions themselves and not the skin around them were cultured. To our knowledge this is the first study of this magnitude to be made of organisms recovered from clean surgical incisions just prior to closure.

EXPERIMENTAL STUDIES

From March through June, 1959, 1020 consecutive clean surgical incisions were cultured. A dry cotton swab in a test tube was sterilized in each surgical pack. The surgeon or his first assistant made the swab around the sides and into the depth of the incision, avoiding the skin, at the end of the operation, just before

TABLE I

Sites of Surgical Incisions, Bacteriological Condition, and Number of Patients Receiving Preoperative Antibacterial Medication

Site of incision	Number	Incisions yielding organisms				Negative incisions			
		Number	%	Premedication	%	Number	%	Premedication	%
Abdomen	578	136	23.5	19	13.9	442	76.5	25	5.6
Arm	38	7	18.4	0	0	31	81.6	0	0
Back	46	9	19.5	3	33.3	37	80.5	3	8.1
Breast or chest	88	17	19.3	1	0.6	71	80.7	11	15.5
Head, neck, face	67	19	28.4	0	0	48	71.6	2	4.0
Leg	111	20	18.0	2	10	91	82.0	3	3.3
Perineum or genitals	14	9	64.2	0	0	5	35.8	0	0
Not indicated	78	12	15.4	1	6.3	66	84.6	3	4.5
Total	1020	229	22.4	26	11.4	791	77.6	47	5.9

the incision was closed. Each swab tube was identified and placed in a refrigerator. Twice daily the tubes were collected and transported to the laboratory in about 20 minutes. Swabs from cases of postoperative infections were handled similarly. Each swab was cultured on the following media: agar containing 5 per cent rabbit blood, no. 110 *Staphylococcus* agar (Difco), and eosin-methylene blue agar. All organisms isolated were tested with Gram stain, observed for hemolysis, and tested for susceptibility to 12 antibiotics by the two dose disc method. The discs were freshly prepared and standardized in our laboratory. Subsequently, some of the cultures that were resistant or moderately resistant to six or more antibiotics were tested for their susceptibility to kanamycin, ristocetin, and vancomycin using the tube dilution technique. Organisms were judged susceptible to kanamycin if inhibited by less than 20 µg./ml., moderately resistant if inhibited by 20 to 50 µg./ml., and resistant if inhibited by greater than 50 µg./ml. For ristocetin and vancomycin the criteria were: for susceptibility, less than 10 µg./ml.; for moderately resistant, 10 to 25 µg./ml.; and for resistant, greater than 25 µg./ml.

Gram-positive cocci were further observed for pigment and coagulase production. Organisms in this paper referred to as *Staph. aureus* are always coagulase-positive strains. The *Staph. aureus* cultures were phage-typed by the modified method² of Blair and Carr³ using 25 test phages.

FINDINGS ON SURGICAL INCISIONS AND POSTOPERATIVE INFECTIONS

Table I shows the operative sites from which swabs were taken. Of the 1020 swabs of clean surgical incisions, 791 (77.6 per cent) were sterile (no organisms were recovered on the culture media employed) and 229 (22.7 per cent) yielded one or more organisms. Of interest is the observation that regardless of the operative site, the percentage of incisions that yielded organisms was relatively constant, between 15 and 28 per cent, except for operations on the perineum or genitals (64.2 per cent). This may be due to the difficulties inherent in preparing sterile operative sites in such cases.

It was also noticed that of the 229 patients with positive incision cultures, 26

TABLE II

Organisms Obtained from Various Incision Sites

Organism	Site								Total
	Abdomen	Arm	Back	B-C	H-N-F	Leg	P-G	U.	
<i>Staph. aureus</i>	10	0	0	1	1	1	1	2	16
Coagulase-negative, pigment positive <i>Micrococcus</i>	13	0	1	2	2	2	2	0	22
Coagulase-negative, pigment negative <i>Micrococcus</i>	102	5	4	14	18	19	8	12	182
<i>Streptococcus</i>	5	1	0	0	0	0	0	0	6
<i>E. coli</i>	2	0	0	0	0	0	0	0	2
Coliform	12	0	1	0	0	1	0	0	14
<i>A. aerogenes</i>	4	0	0	0	0	0	0	1	5
<i>Klebsiella</i>	1	0	0	0	0	0	0	0	1
<i>Proteus</i>	0	0	2	0	0	0	0	1	3
<i>Pseudomonas</i>	3	0	0	0	0	0	0	0	3
<i>Bacillus</i>	7	1	1	2	2	1	0	1	15
Gram-negative coccus	1	0	0	0	0	0	0	0	1
Yeast	1	0	0	0	0	0	1	0	2
Total	161	7	9	19	23	24	12	17	272

B-C = Breast or chest; H-N-F = head, neck, or face; P-G = perineum or genitals; U. = unknown.

(11.4 per cent) had received preoperative antibacterial medication, while of the 791 with negative incision cultures, 47 (5.9 per cent) had not received such preoperative treatment. This difference is probably not significant, since this factor was not controlled by pairing patients or treatments. However, it can at least be concluded that preoperative prophylaxis does not result in any lower incidence of organisms recoverable from operative incisions.

As shown in table II, 272 organisms were obtained (from 229 incisions), including 16 strains of coagulase-positive *Staph. aureus* (from 14 incisions). There were eight postoperative infections, an incidence of only 0.8 per cent. All were from abdominal operations. As shown in table III, swabs of these infections yielded coagulase-positive *Staph. aureus* in 4 cases.

Organisms had been recovered originally from the operative incisions of 4 of the 8 patients in whom postoperative infections developed. Probably the same organism was recovered from both the incision and the postoperative infection in only 2 cases—1 yielded coagulase-negative micrococci (hemolytic, with almost identical antibiograms) and the other, *Staph. aureus* type 80/81/82. The other two incisions that yielded cultures (coagulase-negative micrococci) developed infections in which different organisms were found—a *Staph. aureus* type 80/81/82 and a diphtheroid. No significance is attached to the latter organism.

Postoperative infections developed in 4 cases that had yielded no growth from the original incision swabs. From two of these postoperative infections, *Staph. aureus* type 80/81/82 was recovered (in one instance together with *Escherichia coli*); from one, a mixed culture of *Aerobacter aerogenes* and *E. coli* was obtained; and from another, no organism was obtained. The lack of growth from the last-mentioned swab may have been due to the adverse effect of storage prior to

culturing or, perhaps more likely, to the presence of some antibacterial medicament at the infection site at the time of swabbing.

Survey of Surgical and Nursing Staffs. To locate possible sources and carriers of *Staph. aureus* in the hospital, nasal swabs were taken from 101 of the surgical staff and 152 of the nurses in the nursing units: Coagulase-positive *Staph. aureus* was found in 20 of the former group (20 per cent) and in 38 of the latter group (25 per cent). Strains of phage type 80/81/82 were recovered from 4 of the surgical and 15 of the nursing staff.

Table IV lists all 79 cultures of *Staph. aureus* obtained in this study, together with the phage patterns and antibiograms. For the cultures obtained from the surgical staff, the position or function of each carrier is shown, and for those from the nursing staff, the usual place of duty is given. It is of interest that 8 of the 28 surgeons yielded nasal *Staph. aureus*, as did 6 of the 42 nurses, 3 of the 9 anesthesiologists, 2 of the 19 aides and orderlies, and 1 secretary. The pathologist and the laboratory technician were not carriers.

TABLE III

Cultural Findings on Swabs from 8 Operative Incisions Having Subsequent Postoperative Infections

Case no.	Operative procedure*	Findings on incision culture	Findings on postoperative infection
5	Total abdominal hysterectomy; left oophorectomy	Coagulase-negative <i>Micrococcus</i> (nonhemolytic) MSSS—/SSSSS/R—SS—†	Gram-variable rods (diphtheroid) SSSS—/MSSSS/R—RS—
94	Total abdominal hysterectomy; bilateral salpingo-oophorectomy; appendectomy	Coagulase-negative <i>Micrococcus</i> (hemolytic) MSSS—/SSSSS/S—SS—	Coagulase-negative <i>Micrococcus</i> (hemolytic) MSSS—/SSSSS/M—SS—
294	Right inguinal herniorrhaphy	No growth	<i>Staph. aureus</i> type 80/81/82 SSRSR/SSSR/RSMRS <i>Staph. aureus</i> type 52/80/81/82 SMMSS/SSSR/RSMMS <i>E. coli</i> RSSMM/SRRSM/ SRSSR No growth
324	Total abdominal hysterectomy; right salpingo-oophorectomy; appendectomy	No growth	
495	Cholecystectomy; appendectomy; intestinal adhesions removed	No growth	<i>A. aerogenes</i> SRSR—/ SSRSR/M—SS— <i>E. coli</i> RSSS—/SRMSR/ S—MS—
505	Dilatation and curettage; pelvic laparotomy; left salpingectomy; appendectomy; uterine suspension	Coagulase-negative <i>Micrococcus</i> (hemolytic) SSSS—/SSSSS/S—SS—	<i>Staph. aureus</i> type 80/81/82 SSMSM/SSSR/RSSRS
531	Cholecystojejunostomy; enteroenterostomy	No growth	<i>Staph. aureus</i> type 80/81/82 SSSMM/MSSSR/RSMRS
681	Total hysterectomy; right salpingo-oophorectomy	<i>Staph. aureus</i> type 80/81/82 SSMSR/SSRRS/RSRRS <i>Staph. aureus</i> not typable SSSR/RSSMM/RSRMS	<i>Staph. aureus</i> type 80/81/82 SSMSR/SSSR/RSSRS <i>E. coli</i> RSMMS/SMRRR/SRSMR

* All incisions were abdominal.

† The antibiogram: S = susceptible, M = moderately resistant, R = resistant. The antibiotics are represented in the order: bacitracin, chloramphenicol, chlortetracycline, erythromycin, kanamycin/neomycin, novobiocin, oleandomycin, oxytetracycline, penicillin, polymyxin, ristocetin, streptomycin, tetracycline, vancomycin. A dash indicates that the test referred to was not done.

TABLE IV
Hospital Sources of 79 Cultures of Staph. aureus

Case no.	Phage pattern	Antibiogram*	Surgical staff no.	Function	Phage pattern	Antibiogram
294 inf.	80/81/82	SSRSR/SSSRR/RSRMS	S-39	S	80/81/82	SSMSM/SSSRR/RSRMS
	52/80/81/82	SMMSS/SSSRR/RSMMSS	S-50	RN	80/81/82	SSSS—/SSSRR/R—RM—
358	80/81/82	SSRSS/SSSRR/RSRRS	S-96	An.	6/7/80/81/82	SSRSM/SSSRR/SSRRS
505 inf.	80/81/82	SSMSM/SSSRR/RSRRS	S-48	Sec.	52/80/81	SSSS—/SSSSS/R—SS—
531 inf.	80/81/82	SSSMM/MSSSR/RSRMS	S-28	RN	52/80	SSSS—/SSSSS/R—SS—
681	N.T. (non-hemolytic)	SSSSR/RSSMM/RSRMS	S-24	S	52/81	SSSS—/SSSSS/R—SS—
	80/81/82	SSMSR/SSRRS/RSRRS	S-16	RN	29/52/52A/79/80	SSSS—/SSSRR/R—SS—
681 inf.	80/81/82	SSMSR/SSSRR/RSRRS	S-55	S	47/53/81	SSSS—/SSSRR/R—SS—
54	6/42E/47/75/81/42B/VA ₄	SSSS—/SSSRR/S—SS—	S-42	An.	47/53/VA ₄	SSSS—/SSSSM/R—SS—
611	7	SSSS—/SSSRR/R—MS—	S-72	An.	53/75/VA ₄	RSRRS/SSSRM/MSSRS
829	7	SSSS—/SSSRM/R—MM—	S-92	S	7	SSSS—/SSSRM/R—MM—
134	7/42D/VA ₄	SSSS—/MSSSM/R—MS—	S-44	S	3B	SSSS—/SSSSS/R—SS—
12	29/52/VA ₄	SSSS—/SSSRR/S—SS—	S-8	RN	N.T.	SSMSM/SSSMR/RSRMS
474	79	MSSS—/SSSSS/M—MS—	S-25	RN	N.T.	SSSS—/SSSRR/R—SS—
106	N.T.	SSSS—/SSSSS/M—SS—	S-43	Ord.	N.T.	SMRS—/SSSSS/R—SS—
268	N.T. (non-hemolytic)	RMSMR/SSMMS/RSRMS	S-59	RN	N.T.	SSSS—/SSSSS/R—SS—
			S-77	Aide	N.T.	SSSS—/SSSRR/R—SS—
663	N.T.	SSMSR/RSSMS/RSRRS	S-85	S	N.T.	SSSS—/SSSSM/R—SM—
778	N.T. (non-hemolytic)	SSMSR/MSSRS/RSRRS	S-99	S	N.T.	SSSS—/SSSSS/R—SS—
		SSMSR/MSSRM/RSRRS	S-100	S	N.T.	SSMS—/SSSRS/R—SR—
950	N.T.	MRSS—/SSSSS/R—SS—				
958	N.T.	SSSS—/SSSSS/S—SS—				

Nursing staff no.	Nursing unit	Phage pattern	Antibiogram	Nursing staff no.	Nursing unit	Phage pattern	Antibiogram
N-2	Misc.	80/81/82	SSSS—/SSSRR/R—SS—	N-13	Misc.	47	SSSS—/SSSSS/M—SS—
N-17	1C	80/81/82	MSSSS/SSSRR/RSRRS	N-37	1E	52	MSSS—/SSSSS/R—SS—
N-29	1E	80/81/82	SSRSS/SSSRR/MSRRS	N-108	5F	71	MSSS—/SSSRS/R—SM—
N-33	1E	80/81/82	SSRSM/SSSRR/MSRMS	N-4	Misc.	N.T.	SSSS—/SSSSM/M—SS—
N-50	4F	80/81/82	SSRSS/SSSRR/MSRRS	N-30	1E	N.T.	SSSS—/SSSSS/S—SS—
N-65	4C	80/81/82	MSRMM/SSMRM/RSRMS	N-35	1E	N.T.	SSSS—/SSSSS/R—MS—
N-71	4D	80/81/82	SMRSM/SSSRR/RSRMS	N-40	4E	N.T.	MSSMS/SSSRR/RSRRS
N-79	5F	80/81/82	SMMSM/SSSRR/RSRMS	N-63	4C	N.T.	MSSS—/SSSSS/M—MS—
N-105	5F	80/81/82	SSRSS/SSSRR/RSRMS	N-81	5E	N.T.	SSRSS/SSSRR/MSRMS
N-114	5E	80/81/82	SSMSS/SSSRR/RSRRS	N-84	5E	N.T.	MSSS—/SSSSM/R—SS—
N-80	5E	52/80/81/82	SSRSS/SSMRM/RSRMS	N-85	5E	N.T.	MSMS—/SSSRR/R—SS—
N-25	1C	52/80/81	SSSS—/SSSSS/R—SS—	N-93	4C	N.T.	SSMSM/SSSRR/RSRMS
N-86	5E	81/82	SSSS—/SSSMR/R—RS—	N-112	5E	N.T.	RSRR—/SSSSS/M—SS—
N-124	4F	80/82	SSSRM/SSSRR/RSRRS	N-117	4E	N.T.	SSSS—/SSSSM/R—SS—
N-82	5E	42E/47/75/81/82	SSSS—/SSSSM/R—SS—	N-123	4F	N.T.	MSMS—/SSSRS/S—SR—
N-106	5F	42E/47/75/81/VA ₄	MSSS—/SSSSS/R—SS—	N-126	4C	N.T.	SSSS—/SSSSS/M—MS—
N-67	4D	47/75/81	SSSS—/SSSSM/M—/SS—	N-147	1C	N.T.	SSSS—/SSSSS/M—SS—
N-61	4C	52A/79	SSSS—/SSSSS/R—MS—	N-151	1C	N.T.	SSSS—/SSSSS/R—SS—
N-129	4C	52A/79	SSSS—/SSSSS/R—SS—				
N-91	4F	7/47/53/42B	SSSS—/SSSSM/R—SS—				

Inf. = infection; S = surgeon; RN = registered nurse; An. = anesthesiologist; Sec. = secretary; Ord. = orderly
N.T. = not typable (not susceptible to any of the test phages).
* See table III for key to antibiograms.

The various nursing units produced nasal carriers of *Staph. aureus* in the following proportions of the number tested: unit 1C (4/22), unit 1E (5/18), unit 4C (6/24), unit 4D (2/20), unit 4E (2/16), unit 4F (4/12), unit 5E (8/10), unit 5F (4/13), and from nurses on miscellaneous duty (3/17).

Of the 14 operations that yielded *Staph. aureus* from the incision culture, 11 were attended by personnel tested for nasal carriage, and 8 of these by 1 or more persons from whom *Staph. aureus* was actually cultured. As shown in table V, in 3 instances it appeared that the incision culture and the nasal culture of a staff member present at the operation were probably identical strains of *Staph. aureus*.

In all four of the postoperative infections yielding *Staph. aureus*, the same type was carried in the noses of 1 or more of the nurses in attendance.

Significance of Phage 82. In addition to the usual staphylococcal bacteriophages, phage 82 was also used in typing the cultures of *Staph. aureus*. Formerly known as 52AV, phage 82 was proposed by Comtois and Bynoe⁴ as a substitute for phages 80 and 81, the phages usually associated with epidemic strains. In a previous study of staphylococcal carriers,⁵ phage 82 showed particular promise for identifying antibiotic-resistant strains.

In this study, strains of *Staph. aureus* typed by phage 82 were notably associated with resistance to penicillin, streptomycin, and the tetracyclines. Comparing the 24 type 82 strains with the 55 non-type 82 strains, the incidence of resistance to penicillin was 91.6 per cent in the former group and 21.8 per cent in the latter group; for streptomycin the values were 80 and 16.4 per cent; for chlortetracycline, 46 and 5.5 per cent; for oxytetracycline, 80 and 20 per cent; and for tetracycline, 54 and 13 per cent.

Of all 79 cultures of *Staph. aureus*, only three were resistant to bacitracin and 12 moderately resistant, only one was resistant to chloramphenicol and four moderately resistant, only one was resistant to erythromycin and two moderately resistant, only two were resistant to neomycin and four moderately resistant, none was resistant or even moderately resistant to novobiocin, and only one was resistant to oleandomycin and three moderately resistant. Of 30 cultures tested for susceptibility to kanamycin, ristocetin, and vancomycin because of multiple resistance to other antibiotics, eight were resistant to kanamycin and 10 moderately resistant, while not one was resistant or even moderately resistant to either ristocetin or vancomycin. As might be expected, 59 cultures were resistant to polymyxin, 14 moderately resistant, and only six susceptible.

DISCUSSION

There appears to be little correlation between the organisms found in surgical incisions at the time of closure and those found in postoperative infections. In 225 of 229 cases in which organisms were recovered from incisions, no postoperative infections developed, including 13 of the 14 incisions from which *Staph. aureus* was cultured. Likewise, in 787 of 791 cases in which no organisms were recovered from incisions, no postoperative infections developed. Of the eight postoperative infections, *Staph. aureus* type 80/81/82 was recovered in 4 cases, but in only 1

TABLE V
*Staph. aureus from Incision or Infection as Related to Nasal Carriage
by Surgical or Nursing Personnel*

Case no.	Unit	Source	Phage pattern	Same strain apparently from
268	OR	Incision	Not typable	Nurse S-8
294	4F	Infection	80/81/82	First surgical assistant S-39, nurse N-50
505	5F	Infection	80/81/82	Nurses N-79 and N-105
531	4D	Infection	80/81/82	Nurse N-71
681	OR	Incision	80/81/82	Nurse S-50
681	5F	Infection	80/81/82	Nurses N-79 and N-105
778	OR	Incision	Not typable	Surgeon S-100, nurse S-8

instance had the same strain been isolated originally from the incision swab as well. The only type of *Staph. aureus* found in the infections was the 80/81/82 type.

The finding of the epidemic strain of *Staph. aureus* phage type 80/81/82 in incisions and postoperative infections and in the nares of the surgical and nursing staff indicates that there is a reservoir, albeit small, of potential danger in the hospital. So far the barriers of good technique, sterile practices, good housekeeping, and probably other undeterminable factors have been sufficient to keep the organism within bounds.

In three instances out of 14, it was possible to correlate the finding of *Staph. aureus* in surgical incisions with the attendance of surgical personnel known to harbor the same strains in their nasal passages. In all four postoperative infections in which *Staph. aureus* was recovered, it was possible to postulate an avenue that connected the patient with 1 or more nurses who were nasal carriers of the same strain.

SUMMARY

The relationship between the bacteriology of 1020 consecutive clean surgical incisions and the development of postoperative infection was studied. Organisms were recovered from 229 incisions, including 16 coagulase-positive *Staph. aureus* of phage type 80/81/82, the frequently reported antibiotic-resistant epidemic strain. Eight postoperative infections developed, and *Staph. aureus* type 80/81/82 was recovered from four. There was very little correlation between the organisms found in the incisions at time of closure and those found in postoperative infections, since in only 1 case was the same strain of *Staph. aureus* recovered from the incision and from the postoperative infection that developed. In another case, a coagulase-negative *Micrococcus* was recovered from both the incision and the postoperative infection.

To locate hospital sources of staphylococci, nasal swabs were taken from 101 persons on the surgical staff and 152 on the nursing staff. *Staph. aureus* was found in 20 of the former group and 38 of the latter. Strains of phage type 80/81/82 were cultured from 4 of the surgical and 15 of the nursing staff. Of 14 operations that yielded *Staph. aureus*, 11 were attended by personnel tested for nasal staphylococci and 8 by personnel from whom nasal *Staph. aureus* was recovered. In three instances it appeared that the incision culture and the nasal culture of a staff member present at the operation were identical. In all four of the postoperative infections yielding *Staph. aureus* type 80/81/82, the same strain was carried by 1 or more of the nurses on each patient's ward.

This 18 month old hospital has no overt problem of hospital staphylococcal infections. There is, however, a small reservoir of potential danger, which apparently has been successfully contained by sterile techniques and other effective hospital practices.

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Studies of the *Staphylococcus* in the Upper Respiratory Tract of Patients Receiving Antibiotics in a Rheumatic Fever Convalescence Home

MARK H. LEPPER, JEAN H. PRIEST,* AND ALBERT SIMON

*Department of Preventive Medicine, University of Illinois, Chicago, Ill.,
and the Herrick House, Bartlett, Ill.*

The fact that some strains of staphylococci have become relatively resistant to most of the commonly used antimicrobial agents is accepted. Moreover, it is well recognized that most of these strains are encountered in hospitals and that their occurrence seems to be related to their spread among the personnel and patients. The source of the original resistant strains is believed to be both those that were resistant at the time of the introduction of the antimicrobial as well as those that have become resistant in patients being treated with the agents. The former are recognized to have been the most important as the reservoir from which penicillin and tetracycline resistance arose.

In a study¹ presented at this symposium in 1954 it was reported that analysis of the results in patients being treated with these two agents did not furnish evidence that an increase in resistance to them occurred, but the spread of resistant strains to each of them was the important factor. Since that study was made in hospitalized patients who were treated for short periods of time, a similar analysis of results among patients receiving prolonged prophylaxis in a convalescence home has been made for comparison.

METHOD

All patients were observed during their stay in Herrick House, a rheumatic fever convalescence home. The patients were accepted for admission when their disease had become quiescent enough that rehabilitation could be undertaken. A small number who had signs of activity or who had intercurrent disease were kept in an infirmary; otherwise all of the patients lived in dormitory style. Each child had his own bed and was housed with a varying number of roommates in a manner very similar to that in a large family. The children shared a common dining room, library, play area, and school classes. For the most part, however, they took care of their own hygiene and received little or no nursing care from the personnel. Each patient stayed in the home for a time period adequate to accomplish his rehabilitation, which averaged approximately four and one half months. Throughout the observation period new patients continued to be admitted so that the census was maintained around 20 patients. Since almost all of these patients were recovering from rheumatic fever, they were treated daily with prophylactic doses of antimicrobials.

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* Present address: 12357 14th Avenue, N. E., Seattle, Washington.

TABLE I

Pattern of Isolation of Staph. aureus in Upper Respiratory Tract in Individual Patients

Experi- mental period or group	Medication	Number of patients	Cultures persist- ently negative	Cultures changed from		Cultures intermit- tently positive and negative	Cultures persistently positive	
				Negative to positive	Positive to negative		Phage group changed	No change in phage group
1	Penicillin	8	1	0	1	1*	1	4
2	Penicillin	10	0	2	1	1*	2	4
2	Tetracycline	11	0	1	0	1†	1	8
3	Penicillin	12	0	2	0	2†	4	4
3	No treatment	12	3	1	0	1†	1	6

* No change in phage group.

† Changed phage group.

For the purposes of this study, which extended for 10 months, three therapeutic periods were planned. During the first four months (period 1) a group of 8 patients received buffered penicillin G, 150 mg. twice a day orally. During the second three months (period 2) 10 patients received the same dosage of penicillin, while 11 were given tetracycline, 250 mg. once a day orally if the weight was less than 25 Kg., or twice a day if it was heavier than that. During the last three months (period 3) 12 patients were treated with the same dosage of penicillin and an equal number got no antibacterial medication.

To qualify for inclusion, a patient had to receive the designated medication for the period of observation without other antibacterial drugs. In addition he must not have received "long-acting" penicillin by injection for one month prior to inclusion. Alternate patients who qualified for study were admitted to one of the groups when the comparisons were made.

Separate nose, throat, and nasopharyngeal cultures were made on all study patients and staff members. Since the culture of the nares was almost invariably positive for coagulase-positive staphylococci in the children, only those cultures were used. The patients ranged in age from 5 to 15 years. The personnel were adults more than 20 years of age. They served to indicate the bacterial environment of the study patients. During a given study period no one person was cultured fewer than two times or more than 11. The average number of cultures per patient in one period was seven.

From the cultures a typical colony of *Staphylococcus aureus* was picked to a nutrient broth. If both hemolytic and nonhemolytic varieties or colonies with markedly different pigment formation were present, one of each type was picked to separate tubes of broth. If no colony of *Staph. aureus* was present, one of the *Staph. albus* species was used. In only six instances were no staphylococci identifiable on the plate. Coagulase tests were done by the method of Fisk.² If the culture was coagulase negative, the initial nose culture was re-examined at 48 hours and the procedure just described was repeated. All coagulase-positive cultures were phage-typed according to the method previously reported from this laboratory.³ The minimal inhibitory concentrations of tetracycline and penicillin were determined for all cultures by standard tube dilution methods.¹

TABLE II

Changes in the Minimal Inhibitory Concentration of Penicillin on Serial Isolates from the Same Patients

Fold change	Same phage type isolated serially, patients treated with				Different phage types isolated serially, patients treated with			
	Penicillin	Tetracycline	No antibiotic	Total	Penicillin	Tetracycline	No antibiotic	Total
>20 increase	0	0	0	0	3	0	0	3
12-20 increase	1	0	0	1	1	1	0	1
4-10 increase	5	1	0	6	2	0	0	2
2 increase	6	6	1	13	0	0	0	1
No change	76	36	23	135	0	1	1	2
2 decrease	2	3	0	5	1	0	0	1
4-10 decrease	2	3	1	6	0	0	1	1
12-20 decrease	0	0	0	0	0	0	0	0
>20 decrease	0	0	0	0	0	0	1	1
Total	92	49	25	166	7	2	3	12

RESULTS

In table I it is seen that in all groups at least one third of the patients carried organisms in the same phage group for the entire time of observation. In the tetracycline and no treatment group the persistence of the same phage type was the greatest. When fresh implants occurred these were generally limited to one per patient and the new organisms then persisted for the remainder of the period. Tables II and III illustrate that when new phage types of organisms were acquired, these were generally more resistant to the agent being used than the strains that were replaced. Because of the relatively small numbers, however, these differences lack statistical significance. It can be seen, however, that six of seven strains of a different phage group isolated from patients treated with penicillin were four times or more more resistant to that drug. Similarly, the only two implants that occurred in tetracycline-treated patients were more resistant to it. On the other hand, when the organisms isolated serially were from the same phage group, there was essentially the same distribution of isolates with equal fold increases or decreases in the minimal inhibitory concentration of tetracycline in each treatment group. Moreover, there is essentially the same distribution in all three treatment groups (table III). As seen in table II the change in penicillin minimal inhibitory concentration for serial isolates of the same phage type shows a disproportionate number with a four or more fold increase when compared to the number with a similar fold de-

TABLE III

Changes in the Minimal Inhibitory Concentration of Tetracycline on Serial Isolates from the Same Patients

Fold change	Same phage type isolated serially, patients treated with				Different phage type isolated serially, patients treated with			
	Penicillin	Tetracycline	No antibiotic	Total	Penicillin	Tetracycline	No antibiotic	Total
>20 increase	0	0	0	0	0	0	0	0
12-20 increase	0	0	0	0	0	1	0	1
4-10 increase	1	1	0	2	0	1	0	1
2 increase	14	8	5	27	1	0	0	1
No change	58	31	18	107	5	0	1	6
2 decrease	18	9	2	29	1	0	1	2
4-10 decrease	1	0	0	1	0	0	0	0
12-20 decrease	0	0	0	0	0	0	0	0
>20 decrease	0	0	0	0	0	0	0	0

TABLE IV

Distribution of Minimal Inhibitory Concentrations of Penicillin for Staphylococcal Isolates

M.I.C., units/ml.	All isolates	Mean M.I.C. per phage strain per patient	Isolates in phage group I, M, or I-M		Isolates in phage group II		Isolates in phage group III		Isolates in other or no phage group	
			All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain
>250	161	41	125	27	4	2	18	6	14	6
250	37	9	19	4	2	0	4	3	12	2
125	27	4	14	0	4	2	2	0	7	2
63	13	3	6	0	0	0	2	1	5	2
31	11	0	5	0	0	0	1	0	5	0
16	12	3	6	1	2	0	0	0	4	2
8	4	2	2	1	0	0	1	1	1	0
4	5	3	2	0	0	0	0	0	3	3
2	5	1	3	0	0	0	1	0	1	1
1	2	0	2	0	0	0	0	0	0	0
0.5	1	0	0	0	0	0	0	0	1	0
0.25	0	0	0	0	0	0	0	0	0	0
0.12	0	0	0	0	0	0	0	0	0	0
<0.12	21	12	10	6	3	1	0	0	8	5
Total	299	78	194	39	15	5	29	11	61	23

M.I.C. = minimal inhibitory concentration.

crease among penicillin-treated patients. Similarly, the unequal distribution of fold increases and decreases was different from the finding among the tetracycline-treated and the untreated patients. None of these changes is of sufficient magnitude to be statistically significant, however. Not all of the four or more fold changes were consistently maintained in subsequent isolates.

In tables IV and V are given the minimal inhibitory concentrations of penicillin and tetracycline for each of the isolates from patients in all treatment groups as well as the staff. As is seen, the minimal inhibitory concentration for penicillin is usually greater than 10 µg./ml. or less than 0.12 µg./ml. When there were multiple

TABLE V

Distribution of Minimal Inhibitory Concentration of Tetracycline for Staphylococcal Isolates

M.I.C., µg/ml.	All isolates	Mean M.I.C. per phage strain per patient	Isolates in phage group I, M, or I-M		Isolates in phage group II		Isolates in phage group III		Isolates in other or no phage group	
			All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain	All isolates	No. patients with mean M.I.C./ strain
>250	0	0	0	0	0	0	0	0	0	0
250	1	1	0	0	0	0	0	0	1	1
125	28	8	14	2	0	0	10	4	4	2
63	86	17	71	13	1	1	7	1	7	2
31	49	5	47	5	1	0	1	0	0	0
16	12	3	6	1	5	1	0	0	1	1
8	0	0	0	0	0	0	0	0	0	0
4	1	0	1	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	1	0
1	2	1	0	0	0	0	0	0	2	1
0.5	47	17	15	4	5	2	8	4	19	7
0.25	68	24	40	14	3	1	2	1	23	8
0.12	0	0	0	0	0	0	0	0	0	0
<0.12	4	2	0	0	0	0	1	1	3	1
Total	299	78	194	39	15	5	29	11	61	23

M.I.C. = minimal inhibitory concentration.

TABLE VI

The Relationship of the Minimal Inhibitory Concentrations of Penicillin and Tetracycline for Staphylococcal Isolates

Tetracycline minimum inhibitory concentration, μg./ml.	Treatment group	Penicillin in minimum inhibitory concentration, units/ml.							
		250 or greater		1-249		<1		Total	
		No.	%*	No.	%	No.	%	No.	%
10 or greater	Penicillin period 1	5	19	1	4	0	0	6	22
	Penicillin period 2	15	26	8	14	0	0	23	40
	Penicillin period 3	28	68	1	2	0	0	29	70
	Tetracycline period 2	38	66	14	25	1	2	53	93
	No treatment period 3	14	44	1	3	1	3	16	50
	Staff period 1	8	38	2	10	0	0	10	48
	Staff period 2	13	41	10	31	0	0	23	72
	Staff period 3	11	48	5	22	0	0	16	70
	Total	132	46	42	14	2	(.1)	176	60
	Penicillin period 1	18	67	3	11	0	0	21	78
<10	Penicillin period 2	26	39	9	16	3	5	34	60
	Penicillin period 3	7	17	3	8	2	5	12	30
	Tetracycline period 2*	0	0	4	7	0	0	4	7
	No treatment period 3	11	34	2	6	3	10	16	50
	Staff period 1	7	33	4	19	0	0	11	52
	Staff period 2	2	6	6	19	1	3	9	28
	Staff period 3	2	9	5	22	0	0	7	30
	Total	69	24	36	12	9	3	114	39
	Penicillin period 1	23	86	4	15	0	0	27	100
	Penicillin period 2	37	65	17	30	3	5	57	100
Total	Penicillin period 3	35	85	4	10	2	5	41	100
	Tetracycline period 2	38	66	18	32	1	2	57	100
	No treatment period 3	25	78	3	9	4	13	32	100
	Staff period 1	15	71	6	29	0	0	21	100
	Staff period 2	15	47	16	50	1	3	32	100
	Staff period 3	13	56	10	44	0	0	23	100
	Total	201	70	78	26	11	3	290	100

* Per cent of isolates in treatment period.

isolates from the same patient in the same phage group, the distributions of the mean minimal inhibitory concentrations were not greatly different from that for the individual organisms, a finding that reflects the small changes seen in table II. When the patterns on the different phage groups are compared, it is seen that the group I, M, or I-M, isolates and those in group III have a larger percentage resistant to 250 units of penicillin per ml. or more than was found among the group II, III, mixed group, or nontypable isolates. These differences were statistically significant at the $P < 0.05$ level.

The minimal inhibitory concentrations of tetracycline were distributed into two groups with few intermediate strains. This distribution was seen within all phage groups but the larger proportion of relatively resistance isolates in the group IV, mixed, and nontypable groups is significantly different from the percentage in the other groups ($P < 0.001$).

In table VI the minimal inhibitory concentrations of penicillin were compared with those of tetracycline in the different therapeutic groups. It can be seen that doubly resistant organisms predominated, but there were more penicillin-resistant tetracycline-sensitive organisms than vice versa and fewer doubly sensitive organisms than any other group. It should be noted that during the three penicillin treatment periods there was a significant increase in tetracycline-resistant organisms, particularly among the organisms already resistant to penicillin. All of these differences were significant at a $P < 0.05$ level. In addition only 4 among 57 isolates were inhibited by 10 μg./ml. or less of tetracycline in the group in which this drug was used. On the other hand, during the third period, those patients who did not receive anti-

microbial agents had fewer tetracycline-resistant organisms than did those patients treated with penicillin ($P < 0.05$). The percentage of tetracycline-resistant strains isolated from all patients in the first experimental interval was 22 per cent, a figure significantly lower than that among the total patients in the second period, where it was 67 per cent, or from all patients in the third period, where it was 62 per cent. As can be seen among the personnel, isolates from the first period were also more sensitive to tetracycline ($P < 0.05$).

In spite of the fact that there were few truly sensitive organisms to penicillin, there was an inconsistent variation in the degree of resistance. Thus in the first period there was no significant difference between the isolates from the staff and those from the patients. After the introduction of tetracycline the two treatment groups were identical in respect to penicillin minimal inhibitory concentration, but there were significantly fewer isolates resistant to 250 µg. or more among the personnel. Also, in period three there was no difference between the untreated patients and those who received penicillin. The personnel, however, had fewer of the highly resistant forms ($P < 0.01$).

In table VII data concerned with another source of the changes in the distribution of the minimal inhibitory concentrations are presented. As can be seen, in the first period there was an excess of both tetracycline-sensitive and -resistant strains introduced by new patients over those removed by patients who went home. In the

TABLE VII

Distributions of the Minimal Inhibitory Concentrations of Penicillin and Tetracycline of Isolates from Patients Entering and Leaving the Study

Penicillin, units/ml. Tetracycline, µg /ml.	First isolates from entering patients						Last isolates from discharged patients					
	Penicillin treated, period			Tetracycline treated, period 2	No treatment, period 3		Penicillin treated, period			Tetracycline treated, period 2	No treatment, period 3	
	1	2	3				1	2	3			
Penicillin, 250 or more												
Tetracycline, 10 or more	2	1	5	4	0		1	1	6	2		4
Penicillin, 250 or more												
Tetracycline, <10	1	2	0	0	2		1	3	0	0		3
Penicillin, 1-249												
Tetracycline, 10 or more	2	0	1	1	0		1	1	0	1		0
Penicillin, 1-249												
Tetracycline, <10	3	1	0	1	1		0	1	1	2		1
Penicillin, <1												
Tetracycline, 10 or more	0	0	0	0	0		0	0	0	0		0
Penicillin, <1												
Tetracycline, <1	0	2	2	0	0		0	0	0	0		1
Total penicillin, 250 or more	3	3	5	4	2		2	4	6	2		7
Total penicillin, 1-249	5	1	1	2	1		1	2	1	3		1
Total penicillin, <1	0	2	2	0	0		0	0	0	0		1
Total tetracycline, 10 or more	4	1	6	5	0		2	2	6	3		4
Total tetracycline, <10	4	5	2	1	3		1	4	1	2		5
Total	8	6	8	6	3		3	6	7	5		9

TABLE VIII

Changes in Penicillin Minimal Inhibitory Concentration of Isolates of the Indicated Phage Type Serially Obtained from the Same Patient

Penicillin minimum inhibitory concentration, units/ml., of isolates obtained in																					
Pa- tient	Phage type	Week before penicillin						Week during penicillin												Week after penicillin discon- tinued	
		6	5	4	3	2	1	1	2	3	4	5	6	7	8	9	10	11	12	1	2
1	80, 81	63	63					31	>250		>250		>250								
2	No type				16	31	16	250	125	250	125	125	125		250	125					
3	Gp. II					16	16			250	125	>250									
4	No type	16				2		8	>250		>250		>250	>250	>250		>250	>250	>250	>250	1
5	52, 52A, 80, 81				125	125		16	>250		>250		>250		>250	>250		>250	>250	>250	
	52, 52A, 80								31												
	80, 81						63				>250		>250								
6	81					<0.12															
	52, 52A, 80							>250				>250				>250					
	No type							250													
	80								>250				>250				>250	>250			

second period, among the patients who received penicillin, there was a net loss of one tetracycline-resistant strain and a gain of a sensitive strain, but among the tetracycline-treated patients, there was a gain of 2 new patients with resistant organisms and a loss of 1 patient with sensitive ones. In period three the penicillin-treated patients were weighted by the introduction of new tetracycline-resistant strains, whereas the untreated patients had a net loss of more resistant strains than of sensitive strains.

Among the patients treated with penicillin there were few changes in penicillin minimal inhibitory concentration except for the introduction of four extra moderately resistant strains (1 to 249 $\mu\text{g./ml.}$) in the final period. Isolates from the tetracycline-treated patients showed that there was a net gain of two highly resistant and a loss of one moderately resistant form. The no treatment group had a loss of an excess of 5 patients with the highly resistant forms.

Table VIII presents the data on 6 patients who were the only ones who had serial isolates in the same phage group that showed a consistent change in the minimal inhibitory concentration to either drug. As can be seen, all of these demonstrated increased resistance to penicillin and all were among the penicillin-treated patients. All values and phage types reported in this table were repeated after storage in the deep freeze. Data on the first four patients are consistent with an increased resistance in a given phage type from exposure to penicillin. The pattern of patient 5 may indicate that the isolates were the same throughout or else that they could be an infection with a new strain from closely related phage patterns. In the sixth patient the probability of implantation of a new phage type is great.

To evaluate the significance of the change in the last 2 patients of table VIII, table IX was prepared. From the distribution of the patterns among isolates from different patients it is likely that patients 1 through 11 and patient 18 carried the same or very closely related strains. Patient 5 of table VIII would seem to belong in this group (patient 2 of table IX). Similarly, patients 12 through 15 would seem to have a second strain. Patient 6 of table VIII (patient 13 of table IX) may fall in this group. It is likely, however, that the original penicillin-sensitive isolate,

phage type 81, was from a third strain also found in patients 27 and 28 of table IX. As can be seen, there may have been three other strains in phage group I, namely, those found in patients 16 and 17, in patients 19 through 23, and in patients 24 through 26. It is also clear from table IX that isolates that were lysed only by phages 80 and 81 may have belonged to any of several different strains since they seem to have represented incomplete lysis of several of the full patterns. On the other hand the 80/81 pattern may have represented a distinct strain in some patients.

It must be pointed out that the methods used limited the demonstration of a measurable change in the minimal inhibitory concentration among serial isolates. For example, those strains with only one or no clear tubes (minimal inhibitory con-

TABLE IX
Distribution of Phage Patterns of Serial Isolates Among Patients from Whom Group I, 81, or I-81 Strains Were Obtained

Patient number	Total number of isolates	Number of isolates in each phage pattern							
		52, 52A, 80, 81	52, 52A, 80	52, 80, 81	52A, 80, 81	80, 81	52A, 80	80 81	No type
1	13	9			2	2			
2	12	8			1	3			
3	11	4			2	4		1	
4	11	7			1	3			
5	9	4				4		1	
6	5	5							
7	4	4							
8	3	1				1			1
9	2	2							
10	1	1							
11	1	1							
12	13		6				3	4	
13	9		3					4	1
14	2		1					1	
15	1		1						
16	4			1		3			
17	2			1		1			
18	6				1	4			1
19	3					3			
20	3					3			
21	4					3			1
22	2					2			
23	1					1			
24	1						1		
25	1						1		
26	1						1		
27	5							5	
28	5							4	1
Total	135	46	11	2	7	37	6	11 10	5
Sum of 1-9	70	44			6	17		2	1
Sum of 12-14	24		10				3	9 1	1
Sum of 16-17	6			2		4			
Total 18					1	4			1
Sum of 19-22	12					11			1
Sum of 27-28	10							9	1

TABLE X

Maximal Change in Minimal Inhibitory Concentration of Penicillin Among Organisms of the Same Phage Group Isolated from the Same Patient as Related to Initial Minimal Inhibitory Concentration and Treatment

Treatment group	Initial minimal inhibitory concentration, units/ml.	Fold change					Total
		None or ± 2	Increase		Decrease		
			4-10	12-20	4-10	12-20	
Penicillin period 1	250 or more	3	—	—	1	—	4
	0.1-249	1	2	—	—	—	3
	0.12 or less	—	—	—	—	—	0
Penicillin period 2	250 or more	3	—	—	—	—	3
	0.2-249	1	2	—	—	—	3
	0.12 or less	1	—	1	—	—	2
Penicillin period 3	250 or more	6	—	—	—	—	6
	0.2-249	1	1	—	—	—	2
	0.12 or less	—	—	—	—	—	—
Tetracycline period 2	250 or more	2	—	—	2	—	4
	0.2-249	5	1	—	—	—	6
	0.02 or less	—	—	—	—	—	—
No treatment period 3	250 or more	5	—	—	1	—	6
	0.2-249	1	—	—	—	—	1
	0.12 or less	—	—	—	—	—	—

centration = 250 μ g./ml. or more) could not have had more than a twofold increase demonstrated. Similarly, in those with no growth or growth in only one tube, a decrease of more than twofold could not have been found. Thus, the apparent balance of increases and decreases in table II was biased by the large number of highly resistant strains, as illustrated in table IV. In table X the patients have been placed into three groups according to the sensitivity of their isolates to penicillin. As can be seen, there were 10 patients treated with penicillin in whom increased resistance could have been demonstrated had it occurred. There were 6 such patients in the tetracycline group and 1 in the no treatment group. As previously shown, 6 of the patients in the penicillin-treated groups did show a consistently sustained increased resistance to that drug.

DISCUSSION

Studies in hospitalized populations have repeatedly demonstrated that a high percentage of strains of staphylococci isolated from patients are resistant to antimicrobial agents, whereas those isolated from patients in the outpatient department, or in the community at large, are more susceptible. These findings are the result of several different influences, some of which arise from the manner in which the study is performed and others from the environmental setting. Thus, the frequency of isolation of the same organism from the same patient, or its repeated demonstration in other patients, can be misleading. Moreover, the methods used for measuring changes in reactivity to the drugs are limited when the organism is highly sensitive or resistant. On the other hand, the rate of transfer of organisms from patient to patient, or personnel to patient, the duration of hospitalization, the percentage of patients entering into and discharged from the study, and the pattern of the minimal inhibitory concentrations of the organisms that accompany them all are important determinants of the ultimate balance that is being measured. In

the usual hospital setting we have been unable to evaluate the contribution of all of these factors to the resistance patterns as found. We have reported that most of the resistant forms arise from spread from personnel to patients and vice versa once the patient is in the hospital. The precise origin of the resistant strains was not demonstrated.

In this study in a convalescent home, because of the small patient population, relatively slow interchange of organisms, and ability to randomize therapy, it has been possible to obtain more details about the development and maintenance of a resistant bacterial population in a semiclosed community. In spite of the relatively small number of patients, the comparison of the distribution of minimal inhibitory concentrations among organisms isolated from the patients and personnel is quite similar to that found in many hospitals. This probably reflects the fact that a good many children are admitted from hospitals and, as seen in table VII, may enter with resistant strains, as would be expected. Moreover, at the start of this study, in period 1, when penicillin was being used in the manner that was customary in the hospital, there was much resistance to that drug among the personnel and patients, as would be expected also (table VI). During this first period there was far more resistance than is found outside of hospitals to tetracycline in both the patients and the staff, which probably resulted from the patients being admitted with the doubly resistant organisms and the use of penicillin helping to perpetuate them. Thus there were relatively few major shifts in the minimal inhibitory concentrations of either drug in the penicillin-treated patients (tables II and III). In the second time period, when one half of the patients were treated with tetracycline, there was a marked increase in tetracycline resistance (table VI). Analysis of the groups, however, revealed that among the tetracycline-treated patients this was mainly caused by a persistence of the initially resistant strains (tables I and III), a spread of resistant strains to 2 patients (table III), and a preponderance of resistant strains entering with new patients over those leaving with discharged patients. It is most important to note that both the penicillin-treated patients and the hospital personnel carried flora that was more resistant to tetracycline at this time than during the first period. In the penicillin-treated group this was not accounted for by the acquisition of phage types resistant to the drug or by a preponderance of new patients with resistant forms. It was most likely explained by persistence of the resistant forms in the same patients, which were isolated repeatedly.

The slight reduction of penicillin resistance caused by an increased percentage of moderately resistant isolates from the patients during the second period regardless of drug used was also not well explained by the transfer of strains with lower minimal inhibitory concentrations or by admission of new patients with them. As seen in table X, there were only 3 of 8 patients with highly sensitive organisms in the penicillin group at the beginning of the second period as compared with 4 of 7 in the first. Even though most of these patients did develop more highly resistant isolates (table VIII and X) in both groups, this was more rapid and complete in the first period (table VII, patients 1 and 4) than in the second (table VIII, patients 2, 3, and 6).

In the third period the percentage of tetracycline-resistant isolates continued to rise in the penicillin-treated group and persisted at a 50 per cent level in patients not being treated. It remained high among the staff also. These changes also were

probably not accounted for by the implantation of new strains or admission or discharge of patients as much as by the distribution of organisms at the start of the treatment period. At this time both the penicillin-treated patients and those receiving no therapy showed more penicillin resistance than in period 2. That this was primarily caused by distribution at the start is shown in table X. In one of the penicillin-treated patients (patient 5, table VIII) an increase in resistance occurred and implantation may also have occurred.

From the previous discussion, it is obvious that the small number of patients involved in this study increased the relative importance of bias in group selection over other factors. However, it is equally clear that when similar studies are made on larger groups of patients, it is necessary to consider not only the distribution at the start of the study but the patterns of the organisms among patients entering the study and being discharged from it and exchanges in the patients as well as alteration in sensitivity in a strain in the same patient.

One further point of interest was the difficulty in separating phage types within the same group. As illustrated in table IX, there were probably multiple strains within the group I-M phage patterns. In individual patients the definition of a new strain was much more difficult than in the group as a whole.

SUMMARY

The distribution of penicillin- and tetracycline-resistant staphylococci has been studied in a small number of patients in a rheumatic fever convalescent home.

When penicillin was used as a prophylactic agent there were few truly sensitive organisms isolated from the patients or personnel. Moderately resistant organisms became highly resistant when the drug was used in about half of the patients carrying this particular group of organisms.

When tetracycline was used there was an increase of resistant strains in the entire patient population and staff to that drug. This occurred even though there were relatively few implantations of resistant organisms in treated patients and only a small number of new strains of this type entered this hospital. In a study of this size, however, they were sufficient to account for the results.

The relative importance of the distribution of the minimal inhibitory concentrations of organisms at the beginning of a study, of organisms in new patients and in discharged patients, of exchanges between patients and between patients and personnel, and the development of resistance has been discussed.

Some of the difficulties of separating phage types within phage groups have been demonstrated.

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Triacetyloleandomycin: A Report on Its Use in a General Hospital

M. LEFEBVRE, D. BELIVEAU, R. DESILETS, J. SIMARD, P. E. CHARBONNEAU,
L. LAFORTUNE, AND J. ROY

*Departments of Bacteriology, Surgery, and Medicine, Maisonneuve Hospital,
Montreal, Canada*

Several authors have reported their experiences with triacetyloleandomycin* since the original description of this derivative of oleandomycin by Celmer et al at the Antibiotics Symposium held in 1957.¹⁻³ It has been shown recently that food does not interfere with the absorption of triacetyloleandomycin as it does with that of erythromycin propionate and stearate. Accordingly, when triacetyloleandomycin and erythromycin propionate are given after a meal, the resulting activity in the serum is nearly equal for both preparations.⁴ In a study of staphylococcal infections by Hinton and Wilson, the results of tube dilution sensitivity tests carried out in 200 strains of coagulase-positive staphylococci isolated from hospital infections indicated that about 97 per cent of the strains tested were inhibited by 1.25 µg./ml. of triacetyloleandomycin, a value regularly exceeded in the blood levels on usual dosage schedules.⁵

During the past year we have studied the clinical effect of this compound in about 150 patients with a variety of surgical and medical infections, due largely to coagulase-positive hemolytic staphylococci. In a previous report on the same subject, we have shown that the response to treatment with triacetyloleandomycin was satisfactory when carried out according to sound medical, surgical, and aseptic techniques.⁶ Many of those patients had received other antibiotics, principally penicillin, erythromycin, and chloramphenicol, without obtaining the desired effect. Tolerance in the previously reported series of 66 cases was excellent, and no patient complained of serious gastrointestinal disturbances. The only side effect observed was a mild diarrhea in 2 patients, but this did not necessitate discontinuance of therapy. No allergic reactions were noted and no toxic effect on the hematopoietic system or liver could be detected. No mycotic complications involving the oral cavity or the intestinal tract occurred.

It is the purpose of the present study to report on an additional group of patients similarly treated.

MATERIALS AND METHODS

This series of 81 cases includes patients from both the in- and outpatient departments of the hospital. Most of these patients had localized and soft-tissue infections of various kinds. Wherever possible, a specimen was obtained and the causative organism identified. In the great majority of patients the pathogen was *Micrococcus pyogenes*. Susceptibility tests revealed that most strains were resistant to penicillin,

* The trade name of J. B. Roerig & Co. Division, Chas. Pfizer & Co., Inc., for triacetyloleandomycin is Tao; known in Canada as Olicin.

TABLE I

Clinical Results of Treatment of 81 Patients with Triacetyloleandomycin

Case no.	Diagnosis	Bacteriology	Other antibiotic therapy	Triacetyl-oleandomycin, mg./day	Days of treatment	Results	Untoward effects
1	Bartholinitis		None	300 x 6	6	Good	None
2	Acute bronchitis		None	300 x 6	7	Poor	None
3	Purulent rhinitis		None	150 x 6	7	Good	None
4	Bronchial asthma, secondary infection		None	300 x 6	15	Good	None
5	Sinusitis, bronchitis		None	300 x 6	7	Fair	None
6	Broncho-pneumonia, pleuritis	Beta hemolytic <i>Streptococcus</i>	None	300 x 4	3	Good	None
7	Furunculosis	<i>Staphylococcus pyogenes</i>	Penicillin	250 x 6	6	Good	None
8	Phlegmon	<i>Staphylococcus pyogenes</i>	Penicillin	250 x 6	4	Good	None
9	Left axillary purulent hydradenitis		Penicillin	250 x 4	6	Good	None
10	Urethritis		Penicillin, tetracycline	250 x 6	3	Good	None
11	Furunculosis		None	300 x 4	6	Good	None
12	Furunculosis	<i>Staphylococcus pyogenes</i>	None	300 x 4	8	Fair	None
13	Furunculosis	<i>Staphylococcus pyogenes</i>	Erythromycin	300 x 5	10	Good (recurrence)	None
14	Furunculosis	<i>Staphylococcus pyogenes</i>	None	300 x 5	6	Good	None
15	Furunculosis	<i>Staphylococcus pyogenes</i>	Tetracycline, tetracycline-oleandomycin	300 x 5	12	None	None
16	Furunculosis	<i>Staphylococcus pyogenes</i>	Penicillin	200 x 5	4	Good	Nausea
17	Furunculosis	<i>Staphylococcus pyogenes</i>	Tetracycline, penicillin	300 x 4	4	Fair	None
18	Bilateral axillary hydradenitis	<i>Staphylococcus pyogenes</i>	Penicillin, chloramphenicol	250 x 6	5	Good	None
19	Pulmonary infection during myeloid leukemia	<i>Staphylococcus pyogenes</i>	Chloramphenicol	300 x 5	10	None	None
20	Axillary hydradenitis		Penicillin	300 x 5	8	Good	None
21	Empyema, broncho-pleural fistula	<i>Pseudomonas aeruginosa</i> , <i>Staphylococcus pyogenes</i>	Erythromycin	300 x 5	10	Fair	None
22	Throat infection	Beta hemolytic <i>Streptococcus</i>	Penicillin, sulfonamide	300 x 4	4	Good	None
23	Furunculosis during myeloid leukemia	<i>Staphylococcus pyogenes</i>	Penicillin, erythromycin, chloramphenicol	300 x 5	3	Good	None
24	Endometritis		None	300 x 5	5	Good	None

Table I Continued on Page 757

TABLE I (Continued)

Clinical Results of Treatment of 81 Patients with Triacetyloleandomycin

Case no.	Diagnosis	Bacteriology	Other antibiotic therapy	Triacetyloleandomycin, mg./day	Days of treatment	Results	Untoward effects
25	Hydradenitis	<i>Staphylococcus pyogenes</i>	Penicillin, sulfonamides, tetracycline, chlortetracycline	300 x 5	4	Fair	Nausea
26	Orchitis	<i>Staphylococcus pyogenes</i>	Chloramphenicol, sulfonamides	300 x 5	6	Fair	None
27	Osteitis		Penicillin, erythromycin, penicillin-streptomycin, chloramphenicol, sulfonamides	500 x 3	12	Poor	None
28	Furunculosis	<i>Staphylococcus pyogenes</i>	Erythromycin, penicillin, streptomycin	500 x 3	6	Good	None
29	Furunculosis		Chloramphenicol	500 x 3	6	Good	None
30	Infected incision, purulent discharge	<i>Staphylococcus pyogenes</i>	Erythromycin, kanamycin	500 x 3	10	Poor	None
31	Furunculosis	<i>Staphylococcus pyogenes</i>	Penicillin, streptomycin	500 x 3	6	Good	None
32	Furunculosis	<i>Staphylococcus pyogenes</i>	Erythromycin, penicillin-streptomycin	500 x 3	6	Good	None
33	Furunculosis	<i>Staphylococcus pyogenes</i>	None	250 x 4	6	Good	None
34	Infected ingrown toenail	<i>Staphylococcus pyogenes</i>	Penicillin-streptomycin	250 x 4	4	Good	None
35	Furunculosis		None	500 x 3	6	Good	None
36	Furunculosis		Penicillin, erythromycin, chloramphenicol	500 x 3	4	Good	None
37	<i>Staphylococcus</i> carrier	<i>Staphylococcus pyogenes</i>	None	250 x 4	3	Good	None
38	<i>Staphylococcus</i> carrier	<i>Staphylococcus pyogenes</i>	None	250 x 4	3	Poor	None
39	<i>Staphylococcus</i> carrier	<i>Staphylococcus pyogenes</i>	None	250 x 4	3	Good	None
40	Furunculosis		Penicillin, novobiocin	500 x 3	6	Good	None
41	Viral conjunctivitis		Penicillin-streptomycin	500 x 3	6	Good	None
42	Furunculosis		Oxytetracycline, erythromycin	500 x 3	5	Good	None
43	Paronychia	<i>Staphylococcus pyogenes</i>	Sulfonamide, penicillin, tetracycline	500 x 3	5	Poor	None
44	Furunculosis		Sulfonamide, tetracycline	500 x 3	4	Good	None
45	Furunculosis, paronychia		None	250 x 4	7	Good	None
46	Phlegmon, furunculosis, abdominal wall		None	500 x 3	5	Good	None
47	Hydradenitis	<i>Staphylococcus pyogenes</i>	None	500 x 3	6	Good	None

Table I Continued on Page 758

TABLE I (Continued)

Clinical Results of Treatment of 81 Patients with Triacetyloleandomycin

Case no.	Diagnosis	Bacteriology	Other antibiotic therapy	Triacetyloleandomycin, mg./day	Days of treatment	Results	Untoward effects
48	Furunculosis	<i>Staphylococcus pyogenes</i>	None	500 x 3	9	Good	None
49	Furunculosis	<i>Staphylococcus pyogenes</i>	None	500 x 3	6	Good	None
50	Furuncle	<i>Staphylococcus pyogenes</i>	None	500 x 3	6	Good	None
51	Furuncle	<i>Staphylococcus pyogenes</i>	Penicillin-streptomycin	500 x 3	6	Good	None
52	Furuncle	<i>Staphylococcus pyogenes</i>	Tetracycline, chlortetracycline powder	500 x 3	6	Good	None
53	Furuncle		None	500 x 3	6	Good	None
54	Infected amputation stump		Chloramphenicol	500 x 3	6	Good	None
55	Furuncle		Penicillin (allergy), erythromycin, streptomycin, chloramphenicol	500 x 3	13	Good	None
56	Osteitis		Erythromycin, penicillin, streptomycin	500 x 3	12	Good	None
57	Abscess, cellulitis, elbow		Erythromycin	500 x 3	13	Good	None
58	Osteitis, compound fracture, hand		Penicillin-streptomycin, erythromycin	500 x 3	25	Good	None
59	Furuncle		Erythromycin, tetracycline	500 x 3	6	Good	None
60	Furuncle	<i>Staphylococcus pyogenes</i>	None (allergic to penicillin and tetracycline)	250 x 4	4	Good	None
61	Foreign body infection, hand		Erythromycin	500 x 3	10	Good	None
62	Perianal abscess	<i>Staphylococcus pyogenes</i>	Erythromycin	500 x 3	6	Fair	None
63	Hydradenitis	<i>Staphylococcus pyogenes</i>	Tetracycline	500 x 3	10	Good	None
64	Furuncle		Chloramphenicol	500 x 3	10	Good	None
65	Furuncle	<i>Staphylococcus pyogenes</i>	Erythromycin	500 x 3	6	Fair	None
66	Furunculosis	<i>Staphylococcus pyogenes</i>	Erythromycin, chloramphenicol	500 x 3	6	Fair	None
67	Furuncle		Erythromycin, chloramphenicol	500 x 3	6	Good	None
68	Hydradenitis	<i>Staphylococcus pyogenes</i>	Erythromycin	500 x 3	5	Good	None
69	Furunculosis		Penicillin, sulfonamide	500 x 3	12	Good	None
70	Carbuncle		Penicillin, streptomycin	500 x 3	6	Fair	None
71	Panaris	<i>Staphylococcus pyogenes</i>	None	500 x 3	5	Good	None

Table I Continued on Page 759

TABLE I (Continued)

Clinical Results of Treatment of 81 Patients with Triacetyloleandomycin

Case no.	Diagnosis	Bacteriology	Other antibiotic therapy	Triacetyl-oleandomycin, mg./day	Days of treatment	Results	Untoward effects
72	Furuncle, knee	<i>Staphylococcus pyogenes</i>	None	500 x 3	6	Good	None
73	Furuncle	<i>Staphylococcus pyogenes</i>	Erythromycin	500 x 3	4	Good	None
74	Furuncle	<i>Staphylococcus pyogenes</i>	Chloramphenicol	500 x 3	6	Good	None
75	Ischiorectal abscess		None	500 x 3	10	Good	None
76	Furunculosis	<i>Staphylococcus pyogenes</i>	Tetracycline, penicillin-streptomycin	500 x 3	6	Good	None
77	Abscess, abdominal wall	<i>Staphylococcus pyogenes</i>	Sulfonamide	500 x 4	8	Poor	None
78	Gangrene, osteitis, fourth right finger		Penicillin-streptomycin	250 x 4	6	Fair	None
79	Furuncle		Erythromycin, kanamycin, bacitracin	500 x 3	6	Good	None
80	Furunculosis	<i>Staphylococcus pyogenes</i>	Erythromycin, chloramphenicol	500 x 3	16	Good	None
81	Furuncle		None	500 x 3	6	Good	None

a number also being resistant to one or more of the other antibiotics in common clinical use.

Triacetyloleandomycin was used either in capsule or in oral suspension form. Each capsule supplies 250 mg. of the antibiotic and the oral suspension supplies 150 mg. of oleandomycin activity per teaspoonful. The medication was given according to the severity of the infection and the general status of the patient. Total daily dosage ranged from 1 to 2 Gm. administered in either three or six doses throughout each 24 hours for periods of from 3 to 25 days. Every patient was re-examined at least once after cessation of treatment; the majority were seen twice or three times before final assessment of results. This follow-up period was from two to eight months.

RESULTS

Table I presents the over-all response to triacetyloleandomycin therapy in each case. Of these 81 patients, 62 were considered to show good or adequate response, 11 to show fair response, 6 to show poor response, and 2 failed to respond. Of the 43 cases where it was possible to isolate *M. pyogenes*, results were satisfactory in 38 (88 per cent), poor in 4 (9 per cent), and there was no response in 1 case (3 per cent). Previous therapy with other antibiotics, including penicillin, erythromycin, and chloramphenicol given alone or in combination, was ineffective in 53 patients of this group. Of these, 48 (90 per cent) showed good or fair response to

triacetyloleandomycin. Side effects occurred twice and consisted of nausea in 2 patients. Toxicity was not seen. In only 1 case did the infection recur.

COMMENTS

The results outlined in the preceding section show that triacetyloleandomycin is an effective antibiotic and that it is especially so against pathogenic staphylococci as evidenced by the prompt response to therapy and the minimal number of recurrences during the eight month follow-up. The excellent tolerance to this antibiotic and its lack of toxicity are again demonstrated. This and the effectiveness of triacetyloleandomycin together with the fact that food does not interfere with its absorption make it an ideal antibiotic for use in the treatment of the common infections seen in everyday and hospital practice.⁴

SUMMARY

A clinical study with prolonged follow-up of 81 cases of various types of common infections has confirmed the clinical efficacy of triacetyloleandomycin and its lack of serious side effects and toxicity.

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Gamma Globulin Inhibition in Vitro of Staphylococcal Free and Bound Coagulase

MURRAY M. STREITFELD, BENNETT SALLMAN, AND SEYMOUR M. SHOELSON

*Department of Microbiology, University of Miami School of Medicine, Coral Gables, Fla.,
and the Variety Research Foundation, Miami, Fla.*

Pooled normal human gamma globulin has been observed to exhibit marked therapeutic activity, when used alone or in combination with antibiotics, in infections induced by *Staphylococcus aureus*;¹⁻¹² however, little is known of the mechanism of this protection. We have theorized that gamma globulin may function either to interfere with multiplication of staphylococci, to interfere with the production or release of particular bacterial metabolites essential for virulence, to interfere directly with the action of particular bacterial metabolites essential to virulence, or to activate or supplement some protective mechanism of the host. Various of the metabolic products elaborated by staphylococci have been ascribed etiological roles in the pathogenesis of infection; these metabolites have included the coagulases, the alpha and delta hemolysins, leukocidin, hyaluronidase, staphylokinase, and others.¹³ Investigations of the action of gamma globulin on *Staph. aureus* and its metabolites were undertaken to attempt to add to our understanding of the mechanisms whereby these organisms establish and maintain infection and of the role that gamma globulin may perform in interfering with these processes.

Coagulase activity, since its first description in 1903 by Loeb,¹⁴ has been closely associated with and used as a criterion of pathogenicity for the micrococci; the effect of gamma globulin on the activity of the staphylococcal coagulases was selected therefore as the subject of the first study. Pathogenic staphylococci form two types of material capable of interacting with plasma or fibrinogen. One factor, known as free coagulase, is liberated into the medium during growth, whereas the second, termed "bound coagulase," is apparently part of the bacterial cell wall and is released only by disintegration or autolysis.^{15, 16} There is apparent dissociation of the production of free and bound coagulase, for certain staphylococcal strains isolated from clinical sources were found to produce large amounts of free coagulase but failed to show bound coagulase activity when added to plasma or fibrinogen;^{15, 17, 18} conversely, in vitro subculture of a strain of *Staphylococcus* positive for both free and bound coagulase gave rise to variants that were coated with bound coagulase but produced no free coagulase.¹⁶

Free coagulase is recognized by the formation of a clot when added to a susceptible plasma. Coagulation of fibrinogen occurs readily when a third factor is present, coagulase-reacting factor^{19, 20} or coagulase globulin,²¹ found in the plasma and tissues of human beings and certain animal species.^{19, 22, 23} Coagulase-reacting factor activity has been linked with the prothrombin molecule^{24, 25} or smaller molecules presumably derived from prothrombin, but devoid of prothrombin activity.²⁴ Evidence has indicated that free coagulase as liberated by staphylococci is enzymically

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altered by coagulase activator so that an active fibrinogen-clotting material is formed.^{25, 26} The clotting of susceptible plasma (usually from citrated human or rabbit blood) in the presence of free coagulase is the basis of the tube coagulase test¹⁵ that is today accepted by many workers as a reliable criterion for pathogenicity of strains of *Staph. aureus*.¹³

Bound coagulase, in contrast to free coagulase, is found to act rapidly directly on fibrinogen; no coagulase-reacting plasma factor is required for this interaction.¹⁵ Bound coagulase, also designated as clumping factor, is present on the surface of the majority of pathogenic staphylococci; it absorbs the fibrinogen of human and particular animal species onto the cell surface, causing the cocci to adhere in gross floccules when shaken.^{15, 16, 27} Those animal species whose fibrinogens are acted on by bound coagulase are often not those whose plasmas are clotted by the free coagulase produced by the same cells.¹⁶ The clumping reaction, when it occurs in a susceptible plasma on a slide surface, is termed the "slide coagulase test" and provides a useful screening procedure for the presumptive recognition of pathogenic strains of *Staph. aureus*.^{15, 17, 28, 29} Both free^{13, 15} and bound coagulase^{15, 24} apparently are protein in nature; free^{22, 30, 34} and bound coagulase¹⁵ are antigenic. However, the two coagulases differ not only in occurrence and mode of action, but in antigenic pattern.¹⁶

Evidence presented here indicates that pooled normal human gamma globulin does not inhibit the growth of *Staph. aureus* in vitro, but can interfere markedly with the staphylococcal fibrinogen clumping activity of bound coagulase and with the plasma clotting activity of free coagulase.

MATERIALS AND METHODS

Bacterial Cultures. Twenty-four coagulase-positive strains of *Staph. aureus* were employed in the various studies. These strains included 21 different staphylococcal bacteriophage propagating strains (listed as PS strains in table I), one penicillin-resistant strain (VCH 8), of phage type 44A/42B/52/80/81, isolated from a patient with staphylococcal pneumonia, and two additional penicillin-resistant strains (Giorgio and Rose), selected because of their high degree of virulence for mice.

Gamma Globulin Preparations. Seven lots of poliomyelitis immune globulin, the products of six different commercial manufacturers, were studied. Each product had been processed from large pools of adult human blood and contained 165 mg. (± 1.5 mg.) gamma globulin per ml. One preparation* was available, for experimental purposes, free of preservative; the six commercial preparations† each contained sodium ethylmercurithiosalicylate in a final concentration of 1:10,000. Lots of gamma globulin both free of and containing preservative were studied and com-

* Poliomyelitis immune globulin (human) (lot no. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co., New York City.

† Poliomyelitis immune globulin (human) prepared by: Cutter Laboratories, Berkeley, California (lot no. G0370); Hyland Laboratories, Los Angeles (lot no. 441F11B); Lederle Laboratories Division, American Cyanamid Co. (lot no. 2175-104A7300); Merck Sharp & Dohme Division, Merck & Co., Inc., Philadelphia (lot no. 172 220); Parke, Davis & Co., Detroit (lot no. 046081B); and Pitman Moore Division of Allied Laboratories, Indianapolis, Indiana (lot no. 46948B-1).

pared because: preservative-containing gamma globulin obviously was unsuitable for studies of bacterial growth in the presence of gamma globulin, and sodium ethylmercurithiosalicylate (1:1000) pretreatment of human plasma was reported³⁴ to render plasma unsatisfactory for the tube coagulase test, although not affecting its activity in the slide coagulase test.

Blood Clotting Inhibition Studies with Living Bacteria. Pooled normal human gamma globulin free of antibacterial preservative was studied here for inhibitory action on *Staph. aureus* blood clot formation.

Tests were set up in 7×75 mm. sterile test tubes; a 0.5 ml. aliquot of freshly drawn, oxalated rabbit blood was pipetted into each tube. The tubes were then divided into two lots: assay tubes to each of which there was added 0.1 ml. of 16.5 Gm./100 ml. gamma globulin, so as to give final blood concentrations of 27.5 mg./ml., and control tubes to each of which there was added 0.1 ml. physiological saline. Each set of two tubes (assay and control) was inoculated with 0.01 ml. of a 24 hour Trypticase soy broth (BBL) culture of staphylococci. The sets of tubes were then incubated at 37 C. in a water bath; the tubes were read for clotting of the blood after 1, 2, 3, 4, 5 and 24 hours of incubation.

Duplicate tests were set up, wherein oxalated human blood or Bacto coagulase plasma (Difco) substituted for the oxalated rabbit blood. A single lot number of Bacto coagulase plasma was used for all studies here reported.

As growth controls, assaying the ability of each of the strains of *Staph. aureus* to grow in the presence of gamma globulin, similar sets of tubes were set up, with Trypticase soy broth substituted for rabbit or human blood or Bacto coagulase plasma.

Fibrinogen Agar Assay of Staphylococcal Coagulase Inhibition. A gamma globulin paper strip diffusion test on fibrinogen agar was used here to assay the ability of gamma globulin to inhibit coagulase activity of growing staphylococcal cultures. Fibrinogen agar, whereon coagulase action was demonstrable, was prepared by modification of the method described by Boake.³⁵ Trypticase soy agar adjusted with added agar to contain 4 per cent agar was cooled to 45 C. and diluted with an equal volume of 2 per cent fibrinogen (plasma fraction 1, Armour Laboratories) in saline (sterilized by Seitz filtration). Rabbit serum (2 per cent) was added as the cofactor for free coagulase activity and the whole poured into plates.

The coagulase activity of four strains of *Staph. aureus* (VCH 8, PS 77, PS 80, and Rose) was studied on fibrinogen agar plates. Cotton swabs were used in place of inoculating loops. Each swab was dipped into an 18 hour Trypticase soy broth culture of staphylococci, excess liquid was squeezed off the swab along the upper inner tube surface, and the swab was then used to inoculate the surface of a fibrinogen agar plate along one diameter approximately 2 mm. in width.

Before incubation, each plate had placed upon it centrally, at right angles to the streak of bacterial inoculum, a paper strip saturated with gamma globulin. These paper strips had been prepared by immersing sterile dry strips (0.5×3.5 cm.) of filter paper (Whatman no. 30) in a tube of 16.5 Gm./100 ml. sterile pooled normal human gamma globulin (free of antibacterial preservative), then lightly draining the strips of excess gamma globulin.

The assay plates were incubated at 37 C. for 24 to 96 hours. Growth of the coagulase-positive staphylococcal cultures gave rise to the diffusion of coagulase,

in a relatively steep concentration gradient, in the area adjacent to the bacterial growth. There, interaction of coagulase with fibrinogen and serum activator gave rise to opaque zones of clouding of the medium. At the same time, gamma globulin diffused into the agar media from the paper strips, so that steep gamma globulin concentration gradients were set up throughout the areas adjacent to the strips. As both coagulase and gamma globulin diffused into the agar at right angles to one another, they were free to interact over a relatively wide range of concentration ratios.

Free Coagulase Preparation. A modification of the method used by Barber and Wildy³⁶ was used to prepare solutions containing high concentrations of free coagulase from *Staph. aureus* strains PS 3A, PS 52, PS 77, VCH 8, and Giorgio. Two media were used for coagulase production; these were: heart infusion broth (Difco) (sterilized by autoclaving at 15 lb. pressure 15 minutes) and peptone yeast extract albumin broth consisting of 1 per cent (w/v) peptone (Difco), 0.1 per cent (w/v) yeast extract (Difco), 0.5 per cent (w/v) saline, and 0.15 per cent (w/v) bovine serum albumin (fraction V, Nutritional Biochemicals) (sterilized by Selas filtration). Albumin was used in the latter medium because of the fact that incubation in the presence of albumin^{15,36,38} has been reported to increase the yield of free coagulase for nearly all strains of *Staph. aureus*.

Free coagulase was prepared in single strain lots. An 18 hour broth culture of staphylococci provided the inoculum (loopful) for each of 10 screw-capped tubes (150 × 20 mm.) of heart infusion broth (1 ml./tube). The tubes were incubated at 37 C. in a water bath-shaker, with the shaker speed set at 140 oscillations/minute. After 18 hours of incubation, there was added to each tube 9 ml. of peptone yeast-extract albumin broth (preheated to 37 C.); the tubes were then reincubated and shaken for an additional two hours. The tubes' contents then were pooled and centrifuged; the cells were discarded. Coagulase in the supernatant was concentrated by the addition of 2 volumes of saturated ammonium sulfate. After storage overnight in the refrigerator, the opalescent precipitate was collected by centrifugation, washed once in 60 per cent saturated ammonium sulfate, and taken up in distilled water.

Each coagulase preparation was further purified by a modification of the procedure described by Duthie and Haughton.¹⁶ The aqueous coagulase preparations were adjusted to pH 2.0 (with 1 N hydrochloric acid), then dialyzed for 36 hours at pH 2.0. Small amounts of insoluble material were centrifuged down and discarded. Tests of the coagulase solutions (with barium chloride) for sulfate were negative. The coagulase preparations were adjusted to pH 7.4 (with 0.1 N sodium hydroxide). Certain of the coagulase solutions were sterilized by filtration through fritted glass filters, while other coagulase preparations were preserved by addition of sodium ethylmercurithiosalicylate to a concentration of 1:10,000. Coagulase preparations were stored in the refrigerator (at 4 C.). However, the plasma-clotting potency of the preparations was not well maintained at this temperature, for weekly assays indicated small drops in potency. Therefore, assays of the plasma-clotting potency of a coagulase preparation and the effects of gamma globulin on this assay were performed on individual coagulase preparations within a 24 hour period.

Assay and Standardization of Free Coagulase. Coagulase plasma clotting potency and coagulase inhibition by gamma globulin were assayed on sterile, oxalated

plasma prepared in 25 ml. samples from New Zealand white rabbits. Plasma was stored at 4 C. for 24 to 96 hours before use. The same plasma sample was used for assays of the potency and the inhibition of individual coagulase preparations.

A sterile solution of 0.01 M phosphate in 0.85 per cent saline at a pH of 7.4 was used for dilutions of coagulase. Serial twofold dilutions of each coagulase preparation were prepared in buffer in dilutions ranging from 1:2 to 1:1024; 0.1 ml. aliquots of the undiluted coagulase and of each coagulase dilution then were pipetted to 7 × 75 mm. sterile test tubes. A 0.1 ml. aliquot of plasma was added to each of the 11 tubes and to a plasma-clotting control tube containing only 0.1 ml. aliquots of buffered saline. Final concentrations of coagulase ranged from 1:6 to 1:3072. The rack containing the tubes was shaken and placed in the 37 C. water bath. The tubes were read for plasma clotting (\pm , +, ++, +++, +++++) after 1, 2, 3, 18 and 24 hours of incubation. The highest dilution of coagulase causing +++ or +++++ clotting after 18 hours of incubation was that concentration employed as the constant standard in subsequent assay of inhibition of that coagulase preparation by gamma globulin.

In assays where oxalated human plasma was used in place of rabbit plasma, it was noted that plasma samples from different donors had different abilities to resist clotting by each coagulase preparation, so that higher concentrations of a particular coagulase preparation might be required to clot plasma specimens from different donors. Therefore, each free coagulase preparation was separately assayed against each human plasma sample, in order to determine the concentration of coagulase to be employed as the constant standard in subsequent assays of the effect of gamma globulin on the clotting of the particular plasma sample-coagulase preparation system.

Additional assays were set up, wherein a single lot of Bacto coagulase plasma (Difco) was used in place of oxalated rabbit or human plasma.

Assay of Gamma Globulin Inhibition of Free Coagulase. Fifteen serial twofold dilutions (1:2 to 1:32,768) of each preparation of gamma globulin (16.5 Gm./100 ml.) were prepared (in sterile buffer-saline); 0.1 ml. aliquots of the undiluted gamma globulin and of each gamma globulin dilution then were pipetted into sterile 7 × 75 mm. test tubes. A 0.1 ml. aliquot of coagulase (prepared in the coagulase assay end point concentration) was then added to each of the 16 tubes and to a seventeenth tube (coagulase-clotting control) containing buffer saline in place of gamma globulin. The rack of tubes was shaken, then incubated at 37 C. in a water bath for 30 minutes so as to permit coagulase-gamma globulin interaction to take place. Rabbit plasma then was pipetted in 0.1 ml. aliquots into each of the tubes and into an eighteenth tube (coagulase-clotting control) containing 0.2 ml. buffer. Final assay concentrations of gamma globulin (16.5 Gm./100 ml.) ranged thereby from 1:6 to 1:98,304 (53 to 0.0017 mg./ml.). The tubes were again shaken, reincubated at 37 C., then read for plasma clotting after 18 hours of incubation. The clotting control manifested +++ or +++++ clotting; the assay end point was considered to be the highest dilution of gamma globulin inhibiting this degree of clotting. Minute to very small clots (\pm to ++) were often seen in the tubes containing two- to fourfold concentrations of the assay end point concentration of gamma globulin.

Assay of Gamma Globulin Inhibition of Bound Coagulase. Titrations of gamma

globulin inhibition of bound coagulase were performed by modification of the method described by Duthie.¹⁵ The bound coagulase of *Staph. aureus* strains VCH 8, Giorgio, PS 3A, PS 52A, and PS 77 was studied. Cultures grown 12 to 14 hours at 37 C. on slants of Trypticase soy agar were washed twice with physiological saline and concentrated by centrifugation at 2000 r.p.m. for 15 minutes. The cells were resuspended in physiological saline to a standard density (approximately 3×10^9 cells/ml., as matched to McFarland nephelometer tube no. 10); 0.1 ml. aliquots of this suspension were added to each of 21 tubes (7×75 mm.) for each titration.

Twenty serial twofold dilutions (1:2 to 1:1,048,576) of each preparation of gamma globulin (16.5 Gm./100 ml.) were prepared in 0.85 per cent saline; 0.1 ml. aliquots of the undiluted gamma globulin and of the falling concentrations of gamma globulin then were pipetted into the 21 assay tubes. The tubes were incubated at room temperature (± 26 C.) for 30 minutes. A 0.1 ml. aliquot of freshly prepared 0.2 per cent bovine fibrinogen (plasma fraction 1, Armour Laboratories) then was added to each assay tube, and to a twenty-first tube (bound coagulase-clumping control) containing only 0.1 ml. cells and 0.1 ml. saline. A twenty-second tube (cell control) contained only 0.1 ml. cells and 0.2 ml. saline). Final assay concentrations of gamma globulin (16.5 Gm./100 ml.) ranged, in this assay, from 1:6 to 1:3,145,728 (53 to 0.00005 mg./ml.). The tubes were shaken in a rack in a Kahn shaker for 10 minutes, then read against a dark background using direct lighting and a lens for the degree of clumping (\pm , +, ++, +++). The bound coagulase-clumping control manifested +++ clumping; the assay end point was considered to be the highest dilution of gamma globulin inhibiting ++ or +++ clumping. A minute to very small amount of clumping (\pm to +) was often seen in the tube containing a twofold concentration of the assay end point concentration of gamma globulin.

Human fibrinogen (fraction 1, Merck Sharp & Dohme) was used in place of bovine fibrinogen in an additional assay, by the described procedure, of pooled gamma globulin inhibition of the bound coagulase fibrinogen-clumping reaction. Cells of the Giorgio strain of *Staph. aureus* were utilized for this study.

RESULTS

Table I records the results of assay of gamma globulin inhibition of clotting of rabbit blood by living staphylococci. It can be seen that clotting of oxalated rabbit blood by each of the 24 strains of coagulase-positive *Staph. aureus* was inhibited markedly by gamma globulin acting at a concentration of 27.5 mg./ml.; clotting by 20 of the strains was inhibited for at least 24 hours. When gamma globulin was tested at the lower concentration of 5 mg./ml., blood clotting was delayed for periods varying with the bacterial strain.

In duplicate studies employing Bacto coagulase plasma (Difco) in place of oxalated rabbit blood, clotting of the commercial coagulase plasma similarly was inhibited by gamma globulin.

It is noteworthy that gamma globulin also inhibited the fibrinolytic activity of those staphylococcal strains against which this function of gamma globulin was assayed. *Staphylococcus* strains PS 3C, PS 53, PS 55, PS 75, PS 77, PS 81, VCH 8,

TABLE I

Clotting of Oxalated Rabbit Blood in the Presence and Absence of Gamma Globulin (27.5 mg./ml.) by 24 Different Phage Types of Staph. aureus

<i>Staph. aureus</i>		Hours* required for clotting of:	
Phage group	Phage type	Blood plus saline	Blood plus gamma globulin
I	PS 29	2-3	>24
	PS 52	2-3	>24
	PS 52A/79	9	>24
	PS 80	1	4
II	PS 3A	1	>24
	PS 3B	2	>24
	PS 3C	3	>24
	PS 55	4	>24
III	PS 6	1	>24
	PS 7	2-3	5
	PS 42E	2-3	>24
	PS 47	3	>24
	PS 53	2	4
	PS 54	1	>24
	PS 70	2	4
	PS 75	2	>24
	PS 77	2-3	>24
	PS 42B/47C	2	>24
	PS VA 4	2	>24
	Giorgio VA 4/47/53/54/75/77	1	>24
IV	PS 42D	2	3-4
Miscellaneous	PS 81	1	>24
	Rose 42B/52/80/81	1	>24
	44A/42B/52/80/81 (VCH 8)	1	>24

* Readings taken after 1, 2, 5, 18 and 24 hours of incubation at 37 C.

and Rose were studied here. Rabbit blood clots induced by these strains failed to dissolve after 24 hours of incubation in gamma globulin acting at concentrations of 27.5 or 5 mg./ml.; in contrast, control cultures grown in the absence of gamma globulin showed complete dissolution of clots after this period of incubation at 37 C.

The results of studies of the effects of pooled human gamma globulin on the clotting of oxalated human blood, from five different adult donors, by four different strains of *Staph. aureus* are detailed in table II. It can be seen that the blood samples of one donor (G. K.) were not clotted by the various staphylococcal strains, even in the absence of gamma globulin, within a 24 hour period. However, the blood samples from the other four donors were clotted by the test strains of staphylococci acting in the absence of added gamma globulin; in contrast, in the presence of gamma globulin (27.5 mg./ml.) clotting did not take place within 24 hours.

Gamma globulin did not inhibit multiplication of the 24 test strains of staphylococci. On the contrary, incubation of the organisms at 37 C. in Trypticase soy broth containing gamma globulin (5 or 27.5 mg./ml.) yielded earlier, slightly enhanced growth of the cultures, as estimated by visual inspection of growth turbidity. However, in order to read turbidities, it was found necessary to shake the culture

TABLE II

Clotting of Oxalated Human Blood in the Presence and Absence of Added Gamma Globulin (27.5 mg./ml.) by Four Different Phage Types of *Staph. aureus*

<i>Staph. aureus</i>			Hours* required for clotting of:	
Phage group	Phage strain	Human donor	Blood plus saline	Blood plus gamma globulin
II	PS 3A	M.M.S.	1	>24
		S.M.S.	1	>24†
		J.W.	1	>24†
		J.C.	1	>24†
		G.K.	>24†	>24
III	PS 77	M.M.S.	2	>24
		S.M.S.	>24†	>24
		J.W.	5	>24
		J.C.	2	>24†
		G.K.	>24†	>24
III	Giorgio	M.M.S.	2	>24
		S.M.S.	5	>24
I and III	VCH 8	M.M.S.	2	>24
		S.M.S.	5	>24
		J.W.	2	>24
		J.C.	2	>24†
		G.K.	>24	>24

* Readings taken after 1, 2, 5, 18, and 24 hours of incubation at 37 C.

† After 24 hours of incubation, a tiny clot could be discerned. This is in contrast to the complete clotting seen in the tubes for which clotting is indicated in >24 hours.

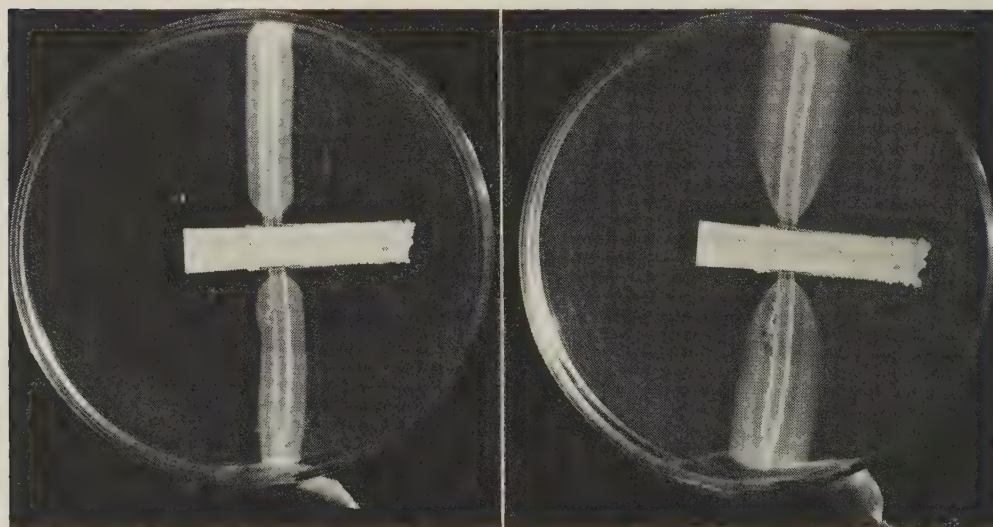


FIG. 1a. (left) Gamma globulin inhibition of the coagulase reaction of *Staphylococcus aureus* (strain VCH 8, phage type 80/81) on a fibrinogen agar plate incubated at 37 C. for 24 hours. Note the absence of inhibition of growth of the staphylococci adjacent to the paper strip saturated with gamma globulin.

FIG. 1b. (right) Same as fig. 1a., after 72 hours of incubation at 37 C. Note the wedge-shaped zones of coagulase inhibition seen at the angles of intersection of the streak of bacterial growth and the paper strip saturated with gamma globulin. Zones of fibrinolysis now are visible adjacent and parallel to the streak of bacterial growth.

TABLE III

Assay End Point Concentrations (mg./ml.) of Gamma Globulin Preparations Causing Inhibition of the Clotting of Oxalated Rabbit Plasma by 9 Free Coagulase Preparations
(Free Coagulase-Inhibition Assay)

Coagulase* from strain	Preservative† in the coagulase	Gamma globulin preparation				
		A‡	B§	C§	D§	E§
VCH 8	Absent	0.43	0.21	0.21-0.43	0.21	0.21-0.43
VCH 8	Present	0.11	—	—	—	—
Giorgio	Absent	0.43	0.86-1.7	0.11-0.43	0.21-0.43	0.21-0.43
Giorgio	Present	0.11	—	—	—	—
PS 3A	Absent	0.43	1.7-3.4	0.21-0.43	0.43	0.43-0.86
PS 3A	Present	0.86	—	—	—	—
PS 52	Present	0.11-0.21	0.43	0.11-0.21	0.11	0.21
PS 77	Absent	0.11	0.43	0.11-0.21	0.11-0.21	0.43
PS 77	Present	0.05	—	—	—	—

* Each of the nine coagulases was prepared individually from stock cultures of the various strains of staphylococci.

† The preservative was sodium ethylmercurithiosalicylate (1:10,000).

‡ Poliomyelitis immune globulin (human) (lot no. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co. This preparation contained no preservative.

§ Poliomyelitis immune globulin (human) preparations of four different commercial manufacturers. The preservative in each preparation was sodium ethylmercurithiosalicylate (1:10,000).

tubes momentarily, to resuspend the bacterial growth, for staphylococcal cultures growing in the presence of gamma globulin were found clumped at the tubes' bottoms, in contrast to the control cultures' diffuse growth throughout Trypticase soy broth free of gamma globulin.

Figure 1 depicts the results of a fibrinogen agar assay of coagulase inhibition by gamma globulin; these findings are typical of those observed with the four coagulase-positive strains of *Staph. aureus* studied. It can be seen that bacterial multiplication was not inhibited by gamma globulin. However, clear wedge-shaped zones, indicative of gamma globulin inhibition of coagulase clouding of the fibrinogen agar in these zones, were present at the angles of intersection of the paper strip and the bacterial growth. The presence of these clear wedge-shaped zones was interpreted as indication that gamma globulin either inhibited production of coagulase by the staphylococci, so that ever smaller amounts of coagulase were formed in the presence of increasing amounts of gamma globulin, and/or inhibited coagulase activity in specific concentration ratios, so that low concentrations of gamma globulin inhibited the activity only of low concentrations of coagulase, while the activity of higher concentrations of coagulase was inhibited only by relatively higher concentrations of gamma globulin. Studies with purified coagulase, free of bacterial cells, were undertaken to elucidate this latter problem.

Table III indicates that each of the five gamma globulin preparations assayed inhibited free coagulase from coagulating oxalated rabbit plasma. The activities of the coagulases of the staphylococcal strains (VCH 8, Giorgio, PS 3A, PS 52, and PS 77) assayed were inhibited by low minimal concentrations of gamma globulin, ranging from 0.05 to 3.4 mg./ml. The gamma globulin preparations varied somewhat in anticoagulase potency, so that four of the preparations (A, C, D, and E) appeared more effective than did the fifth (B) and gave a relatively narrow range of assay end points, 0.05 to 0.86 mg./ml. No marked differences were observed in

TABLE IV

Assay End Point Concentrations (mg./ml.) of Gamma Globulin Causing Inhibition of the Clotting of Oxalated Human Plasma by 2 Free Coagulase Preparations

Coagulase* from strain	Number of plasma samples†	Gamma globulin,‡ mg./ml.
Giorgio	4	0.86
VCH 8	3	0.11–0.86

* The coagulase preparations contained sodium ethylmercurithiosalicylate (1:10,000) as preservative.

† Four human donors each contributed one plasma sample.

‡ Poliomyelitis immune globulin (human) (lot no. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co. This preparation contained no preservative.

the activity of individual gamma globulin preparations against the various coagulases here assayed.

Studies designed to ascertain the effect on the anticoagulase assay of gamma globulin of the sodium ethylmercurithiosalicylate used as preservative (in a 1:10,000 dilution) for coagulase and or gamma globulin indicated (table II) that the assay end points were affected only slightly or not at all by the preservative in the final concentrations obtained in the assays. A sodium ethylmercurithiosalicylate control assay, wherein a solution (1:10,000) of the preservative in buffer saline was assayed alone (in place of gamma globulin) for coagulase-inhibitory effect in the standardized anticoagulase assay, revealed that the preservative caused partial inhibition of coagulase when used in a final assay concentration of 1:30,000 and no inhibition at higher (twofold) dilutions.

Pooled gamma globulin also inhibited the clotting of oxalated human plasma by partially purified free coagulase preparations (table IV). In these assays, the activities of the free coagulases of the virulent VCH 8 (phage type 80 81) and Giorgio strains of staphylococci were obtained by low concentrations of gamma globulin, ranging from 0.11 to 0.86 mg./ml.

Bound coagulase also was inhibited by gamma globulin. Table V reports the results of assays of the seven gamma globulin preparations for inhibition of bovine

TABLE V

Assay End Point Concentrations (mg./ml.) of Gamma Globulin Preparations Causing Inhibition of Clumping in Fibrinogen of 5 Strains of Staph. aureus (Bound Coagulase-Inhibition Assay)

<i>Staph. aureus</i> strain	Gamma globulin preparation						
	A*	B†	C†	D†	E†	F†	G†
VCH 8	0.013	0.013	0.0067	0.027	0.0067	0.11	0.013
Giorgio	0.013	0.0067	0.013	0.0067	0.0034	0.0034	0.0067
PS 3A	0.013	0.023	0.0067	0.0067	0.054	0.027	0.0067
PS 52A	0.0067	0.0067	0.013	0.0067	0.0067	0.013	0.0067
PS 77	0.0067	0.0067	0.0067	0.0067	0.0034	0.0034	0.0067

* Poliomyelitis immune globulin (human) (lot no. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co. This preparation contained no preservative.

† Poliomyelitis immune globulin (human) preparations of six different commercial manufacturers. The preservative in each preparation was sodium ethylmercurithiosalicylate (1:10,000).

fibrinogen-clumping of five strains of *Staph. aureus*; it can be seen that minimal inhibitory concentrations of gamma globulin varied from 0.0034 to 0.054 mg./ml. Each gamma globulin preparation varied slightly from the other preparations in anti-bound coagulase activities demonstrated against the different strains of staphylococci. Substitution of human for bovine fibrinogen in the assay of the anti-bound coagulase fibrinogen-clumping reaction by pooled gamma globulin indicated that this in vitro effect of *Staph. aureus* (Giorgio strain) and its metabolites on organic materials wholly derived from human sources was similarly inhibited.

Comparison of the anti-bound coagulase assay titers obtained with the preservative-free preparation of gamma globulin and the six gamma globulin preparations containing sodium ethylmercurithiosalicylate indicated that all antagonized the activity of bound coagulase to approximately the same extent. Control assays set up so that the preservative-free lot of gamma globulin was assayed in parallel with the same preparation containing ethylmercurithiosalicylate (1:10,000) gave identical end points for both assays. It is believed, therefore, that the ethylmercurithiosalicylate used as a gamma globulin preservative did not appreciably alter anti-bound coagulase titers when diluted (together with the gamma globulin) in the standardized assays.

DISCUSSION

Experimental evidence presented in this paper has indicated that pooled normal human gamma globulin interferes in vitro both with the plasma clotting activity of free coagulase and with the fibrinogen clumping activity of bound coagulase. It is presumed that a similar inactivation may occur in vivo; such action, however, has not been demonstrated. Nor are the contributions of the coagulases to staphylococcal virulence well established. However, if coagulase modifies staphylococcal virulence at least in part, then, it is important to understand the effects that the staphylocoagulases may exert both in vitro and in infection.

Presumptive evidence has been obtained¹⁸ that the coagulase-inhibiting components of gamma globulin are antibody in nature in the findings that the activity of gamma globulin against bound coagulase could be removed by absorption with staphylococci containing the homologous coagulase, leaving unaltered the anti-free coagulase activity. Attempts at absorptions of antibodies to free coagulase by several methods, however, were not successful.

Antibodies to free coagulase have been found in the sera of normal human beings^{22, 30, 32, 39, 40} as well as being produced by immunization of animals.^{22, 41, 42} Antisera against free coagulase showed no inhibitory action on bound coagulase.^{15, 33} Several studies on the immunological specificity of the cell-free coagulases have reported the existence of two antigenically distinct staphylocoagulases, with a third related to both;³² four different antigenic types of albumin-extracted coagulase³³ and a close relationship between the bacteriophage typing group of the staphylococcal strain and the antigenic specificity of its free coagulase.³⁶ In the last study, staphylocoagulases of phage types 3A and 42E differed antigenically from those of other group II and group III strains respectively. In our studies, pooled human gamma globulin inhibited the coagulases of phage groups I, II, and III and types 3A and 42E. It is also noteworthy that the same normal human gamma

globulin inhibited the free and bound coagulases of the highly contagious, virulent, and penicillin-resistant *Staph. aureus* phage type 80/81, which has been of so much concern in infections of hospitalized patients,⁴³ as well as the staphylocoagulases of other human and animal virulent phage types.

Although antibodies to free coagulase are specific for the coagulase of certain staphylococcal strains, it has been suggested¹⁵ on the basis of the available evidence that the antibody for bound coagulase is nonspecific. The experimental data recorded in the present study indicate that the pooled human gamma globulin contained antibodies against the bound coagulase of each of the five strains of staphylococci assayed.

The demonstrated anticoagulase activity of gamma globulin in vitro raises the question as to whether it may exert a similar action in vivo. It has been reported⁴⁴ that rabbits experimentally infected with coagulase-positive staphylococci could be protected passively with human sera containing free coagulase-inhibiting substances. Studies³⁵ on rabbits actively immunized with free coagulase have indicated that these animals had increased resistance to intravenous challenge with the homologous strain of coagulase-positive staphylococci; no protection was conferred against challenge with a toxigenic coagulase-negative variant strain of *Staphylococcus*. Virulence of coagulase-positive staphylococci for mice was diminished by treating the bacteria, before inoculation, either with anticoagulase prepared from rabbits immunized with coagulase, or with human serum containing coagulase-inhibiting antibodies. These effects appeared to be related to inhibition of coagulase activity, since they were not produced by alpha antitoxin or staphylococcal agglutinating serum and were not demonstrable against the coagulase-negative variant strain of *Staphylococcus*. Pooled human gamma globulin, by virtue of its anticoagulase activity, might be expected to exert similar protective effects in vivo against *Staph. aureus*.

A knowledge of the contribution of coagulase to staphylococcal virulence might clarify the role of gamma globulin as a protective agent in staphylococcal disease. Despite extensive investigation,¹³ the coagulase-virulence interrelationship is still incompletely understood. In an attempt to explain the mechanism of formation of the focal abscesses characteristic of staphylococcal infections, a critical examination was recently made²³ of the attractive hypothesis that coagulase, by assisting in the deposition of a fibrin barrier, impeded the elimination of *Staph. aureus* by cellular defense mechanisms, while allowing the multiplication of the bacteria in the inflamed tissues. Titers of coagulase-reacting factor concentrations in the sera of human beings in different age groups were related to the different types of staphylococcal infectious processes commonly found at various ages. Low titers of coagulase-reacting factor were the rule in infants, where abscess formation is relatively uncommon but bacteremia and osteomyelitis are frequently observed; however, relatively high serum titers of reacting factor were usually encountered in the adult age group, where abscess development in staphylococcal infection is commonly encountered. In another phase of the same study, it was found that type-specific anticoagulase in the sera of monkeys partially protected the animals against abscess formation by a homologous, but not a heterologous, free coagulase type strain of staphylococci. Other investigators^{45, 46} have reported that coagulase-producing strains of staphylococci were, in a coagulable medium, less readily phagocytized

in vitro by human leukocytes than were strains that produced no coagulase; the ability of coagulase-positive staphylococci to inhibit phagocytosis was correlated with the deposition of fibrin on the surface of the bacteria and the subsequent clumping of the organisms. Studies⁴⁷ in animals experimentally infected with *Staph. aureus* further indicated that the in vivo elaboration of coagulase could delay, by the initiation of deposition of a fibrin barrier about the cocci, the phagocytosis of the bacteria. Another facet in the relationship of coagulase to virulence was revealed by the finding that while coagulase-positive staphylococci resisted the antibacterial action of human defibrinated blood⁴⁸ or serum,⁴⁹⁻⁵¹ coagulase-negative strains were readily inhibited. It has been reported^{50, 51} that partially purified free coagulase preparations from cultures of coagulase-positive, serum-resistant staphylococci were effective in neutralizing the bacteriostatic activity of normal human serum for coagulase-negative staphylococci. The protective role of pooled human gamma globulin in the coagulase-virulence interrelationship awaits further experimental clarification.

It has been emphasized⁹ that many antigenic staphylococcal metabolites in addition to coagulase may mediate staphylococcal virulence. Thus, pooled human gamma globulin is a complex material that may contain antibodies not only to the staphylocoagulases, but to a wide variety of the somatic antigens and metabolites of staphylococci and other bacteria in addition to its known antiviral components.^{52, 53} Studies of the effects of gamma globulin on certain of these factors are currently in progress.

SUMMARY

Pooled normal human gamma globulin, alone or in combination with antibiotics, has been found to exert marked therapeutic activity in *Staph. aureus* infections. In seeking to understand the mechanism of this protection, it was decided to investigate the effect of gamma globulin on staphylococcal coagulases, both free and bound. The results of this in vitro study of gamma globulin action were: the coagulation of oxalated rabbit and human blood by many phage types (groups I, II, and III, and strains of type 80/81) of *Staph. aureus* was markedly inhibited; the clotting of rabbit and human plasma by five partially purified preparations of free coagulase was inhibited by high dilutions of gamma globulin; the bovine and human fibrinogen-clumping activity of bound coagulase was prevented by very small amounts of gamma globulin; six different commercial preparations of pooled human gamma globulin were found to be equally effective as inhibitors of free and bound coagulase; and multiplication of 24 bacteriophage type strains of *Staph. aureus* was not prevented despite the effective coagulase-inhibitory action of the gamma globulin. These significant effects of gamma globulin on staphylocoagulases may account in part for its demonstrated therapeutic efficacy.

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Furaltadone, Pharmacological and Clinical Studies*

WILLIAM R. McCABE, GEORGE GEE JACKSON, AND VERA M. KOZIJ

*University of Illinois Research and Educational Hospitals and Department of Medicine,
University of Illinois, College of Medicine, Chicago, Ill.*

Various nitrofurans have been shown to inhibit staphylococci in vitro,^{1,2} but they have had little or no clinical usefulness in the treatment of systemic infections because of the failure to attain antibacterial levels in the serum after oral administration. A new nitrofuran, 5 morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidone, with the generic name furaltadone, has antistaphylococcal activity in vitro and was beneficial in experimentally induced staphylococcal infections in animals. In human beings, after oral ingestion, the drug was detected in the recipients' serum.³ The present report concerns bacteriological, pharmacological, and clinical observations on the use of furaltadone in patients having staphylococcal infections.

METHODS AND MATERIALS

Laboratory. The sensitivity of staphylococci and micrococci to furaltadone was determined by the serial twofold tube dilution method. The tube containing the least amount of drug that had no visible growth after 24 hours of incubation was considered the minimal inhibitory concentration. The minimal bactericidal concentration was considered the smallest amount of antibiotic that inhibited visible growth at 24 hours and showed no regrowth on antibiotic-free medium from 0.05 ml. of inhibited culture. Varying inocula sizes were tested for their influence on the observed sensitivity.

Some bacterial sensitivity determinations also were performed by the paper disc method. The diameter of the zone of inhibited bacterial growth surrounding a 50 µg. disc was measured in mm. and correlated with the sensitivity determined by the tube dilution method.

Bacterial growth curves in the presence of the drug were obtained for five strains of staphylococci. Aliquots from each of a series of tubes containing decreasing concentrations of antibiotic and a standard inoculum of the test strain were studied at 3, 6, 24, and 48 hours after inoculation.

Furaltadone levels in sera and body fluids were determined by the colorimetric method of Buzard et al.⁴ In order to make readings in the optimum range and to detect concentrations of less than 5 µg./ml., duplicate determinations were made after the addition of 10 µg. of furaltadone to the test serum.

Clinical. Patients with staphylococcal infections were treated with varying oral dosage schedules of furaltadone. The infecting *Staphylococcus* and the strains isolated on discontinuation of treatment or at the time of bacteriological relapse were obtained, if possible. Coagulase tests, bacteriophage typing, and sensitivity

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TABLE I

Tube Dilution and Filter Paper Disc Sensitivity of Staphylococci to Furaltadone

Tube sensitivity, minimal inhibitory concentration, $\mu\text{g./ml.}$	Number of strains		Disc sensitivity, diameter of zone of inhibition, mm.	
	Clinical series	Laboratory series	Mean	Range
1.0	0	1	23	—
1.9	0	1	22	—
3.9	2	19	22.9	19–33
7.8	13	16	21.4	19–27
15.6	4	1	21.0	—

tests were performed on all strains. Random blood specimens throughout the course of treatment were obtained at two, four, or six hours after an oral dose of 200 mg. of furaltadone. Serial hemograms and liver and renal function studies were obtained on all patients.

RESULTS

Strains of staphylococci of diverse phage types and some with widely different sensitivities to all the commonly available antibiotics were tested for their sensitivity to furaltadone. The results are given in table I. Of 38 selected laboratory strains, 35 had a minimal inhibitory concentration in the range from 3.9 to 7.8 $\mu\text{g./ml.}$ Two strains were more sensitive and one was more resistant. In concomitant disc sensitivity, zones of 19 to 33 mm. of inhibited growth surrounded a 50 $\mu\text{g.}$ disc. The size of the zones showed sufficient overlap to make this method only an estimate of sensitivity and not well correlated with the tube dilution values. Also included in table I are the sensitivities of 19 strains of staphylococci isolated from patients with staphylococcal infections prior to treatment with furaltadone. Fifteen of them or 79 per cent of the strains isolated from clinical infections were inhibited in vitro by 7.8 $\mu\text{g./ml.}$ The remainder of the clinical strains required 15.6 $\mu\text{g./ml.}$ for inhibition. Studies with various inoculum sizes of 10^2 , 10^4 , and 10^6 bacteria/ml. showed only a twofold increase or decrease in the inhibitory concentration of the antibiotic with a two logarithm increase or decrease in the inoculum size.

Figure 1 is a graph of composite bacterial growth curves for five strains of staphylococci in the presence of increasing antibiotic concentrations. The observed minimal inhibitory concentrations for the test strains were 7.8 $\mu\text{g./ml.}$ at 24 hours and 7.8 to 15.6 $\mu\text{g./ml.}$ at 48 hours. It is apparent that the minimal inhibitory concentration prevented bacterial growth and at 24 hours had produced a one logarithm decrease in the number of bacteria inoculated. Antibiotic concentrations less than the minimal inhibitory concentration produced only slight inhibition of bacterial multiplication. A drug concentration two- to fourfold greater than the minimal inhibitory concentration decreased the bacterial count by four or five logarithms, and only 100 or less viable cells/ml. were present after 24 hours. The sensitivity of our method made it impossible to measure less than 20 bacteria/ml. Continued incubation after 24 hours resulted in an increase in viable bacteria with either 7.8 or 31.2 $\mu\text{g./ml.}$ This increase in bacterial count indicates either drug degradation or regrowth of resistant clones.

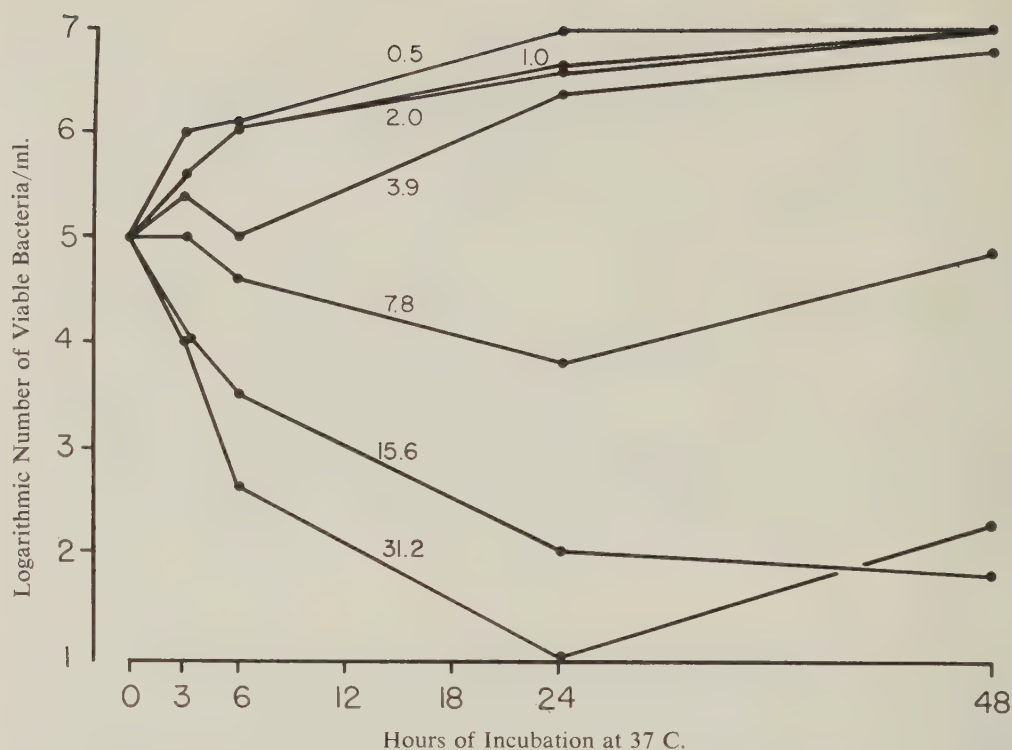


FIG. 1. Mean growth of five strains of staphylococci in varying concentrations of furaltadone, $\mu\text{g./ml.}$

To test these factors, the activity of the drug was measured after it had been incubated at 37 C. for 6, 24, 48, and 96 hours. The drug also was incubated with cell-free staphylococcal filtrate and with a whole culture of staphylococci. The results of these studies are presented in figure 2. The decrease in antibacterial activity is plotted against the period of incubation in each system. No change in activity was observed at 96 hours incubation of the drug alone. Only one half the antibacterial activity originally observed was still present after 24 hours' incubation with cell-free staphylococcal filtrate. No further change was observed after additional

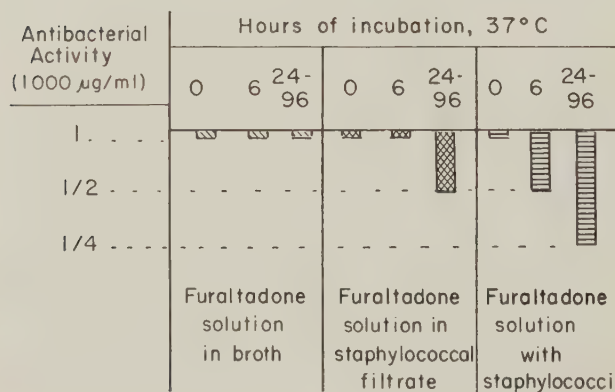


FIG. 2. The activity of furaltadone after incubation.

TABLE II
*Type of Infection in 23 Patients Treated
with Furaltadone*

	Number*
Bacteremia	11
Deep wound infections or abscesses†	8
Pneumonia or empyema	4
Pyarthrosis	2
Wound infection	1
Endocarditis	1
Meningitis	1
Osteomyelitis	1

* Several patients had more than one site of infection.

† Includes 3 patients who developed bacteremia during treatment for a localized staphylococcal infection.

incubation up to 96 hours. If the drug was incubated with a culture of staphylococci for six hours and then rendered bacterial free by filtration, the antibacterial activity was decreased by one half. Twenty-four to 96 hours incubation with staphylococci reduced the antibacterial activity of furaltadone to one fourth of that which was originally present.

In vitro production of resistance to furaltadone was tested by incubation of five strains of staphylococci in subinhibitory concentrations of furaltadone. Twelve to 13 such passages produced a stepwise increase in resistance to furaltadone that suggested obligatory multiple-step mutation. This usually occurred in two- to four-fold increases in resistance until the organisms were resistant to 250 µg. or more of furaltadone. No attempt was made to induce resistance beyond this level.

The type of infection treated with furaltadone among patients is given in table II. Several patients had more than one site of infection; this was most often septicemia associated with a local infection. The five instances of deep wound infections all represent infections that occurred after insertion of a metallic hip prosthesis. All of the patients had some anatomical disease in addition to staphylococcal infection. Two dosage regimens were used. Initially, the dosage was 200 mg. every six hours and this was later increased to 200 mg. every four hours because of the low drug levels obtained with the smaller dosage.

Blood levels observed two, four, and six hours after oral administration are shown in figure 3. The solid dots represent a total daily dosage of 800 mg. and the open dots represent daily intake of 1200 mg. As can be seen, 55 per cent of the specimens obtained two hours after ingestion contained no appreciable amount of furaltadone. Four and six hours after a dose 77 per cent and 88 per cent of the sera respectively had no demonstrable level. Four of the 18 patients had no level in any of the sera tested. Three had detectable levels at each time interval and 5 at two and four hours but not six hours after the dose. Drug levels in 5 patients who received 1200 mg. of furaltadone were no more frequent and no higher than in the patients who received 800 mg. a day. Only 25 per cent of the specimens at two hours, 14 per cent at four hours, and none at six hours were

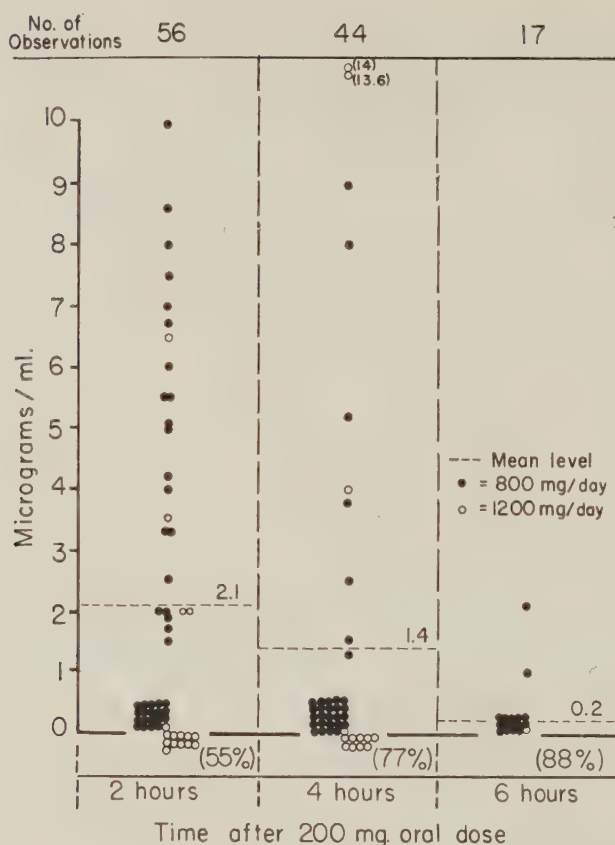


FIG. 3. Blood levels observed two, four, and six hours after oral administration of furaltadone.

more than 3.9 $\mu\text{g./ml.}$, which was the lowest in vitro inhibitory concentration for any of the infecting strains.

Results of treatment with furaltadone, in general, were disappointing and are outlined in table III. Only 2 of 8 patients with abscesses or wound infections attained a clinical and bacteriological cure. Three of the remaining 6 patients in

TABLE III

Results of Furaltadone Treatment of Staphylococcal Infections in 23 Patients

Disease	Number of patients	Clinical result		Bacteriological result	
		Cure	Failure	Cure	Failure
Wound infection of abscesses*	8	2	6	2	6
Bacteremia or endocarditis	8	6†	2	7	1
Pneumonia or empyema*	5	0	5	0	5
Meningitis or osteomyelitis	2	0	2	0	2

* Three patients developed bacteremia during furaltadone treatment for localized infection. These are not included in tabulation of bacteremia cases.

† Two patients relapsed after initial clinical and bacteriological cure but are tabulated as clinical and bacteriological cures.

TABLE IV
Adverse Reactions Associated with Furaltadone

Type of reaction	Number
None	9
Nausea or vomiting	8
Eosinophilia	7
Skin eruption	2
Granulocytopenia	1
Azotemia, nephropathy	1
Allergic reaction	1

this group developed a bacteremia with staphylococci during treatment with furaltadone. The best results were obtained in the treatment of blood stream infection. Six of 8 patients demonstrated an initial clinical and bacteriological response. Two of them relapsed shortly after the discontinuation of furaltadone, with recurrent septicemia in one instance and recurrence of a purulent joint effusion of the elbow in the other. The one instance of sterile blood culture associated with clinical failure was in a patient with micrococcal endocarditis who had a persistent febrile course and ulcerative valve vegetations at necropsy despite repeatedly negative premortem blood cultures. None of the 7 patients with pneumonia, empyema, meningitis, or osteomyelitis were clinically or bacteriologically cured. If a bacteriophage typable strain of *Staphylococcus* was recovered before treatment, the same type invariably was present in specimens obtained after treatment from patients in whom treatment failed or relapse occurred.

Reactions temporally related to the administration of furaltadone were observed in 14 of 23 patients and are shown in table IV. Nausea and vomiting occurred in about one third of the patients. Eosinophilia to the extent of 6 to 14 per cent of the total leukocyte count also was observed in one third of the patients. These bore no relation to one another.

In four instances furaltadone was discontinued because of potentially severe reactions. One of these patients developed sudden onset of chills, fever, ringing in the ears, myalgia, pruritus, and nausea and vomiting after 13 days of furaltadone treatment. These effects subsided within 24 hours after discontinuation of the drug and were reproduced three days later by the administration of three doses of 200 mg. each of furaltadone. One patient developed neutropenia. The total leukocyte count on the fifth day of treatment with furaltadone was 1700; no polymorphonuclear leukocytes were present in the differential count. This patient had received ristocetin, 2.0 Gm./day, for six days immediately prior to treatment with furaltadone. A blood count on the day treatment with ristocetin was terminated showed 6350 leukocytes/cu. mm. of which 52 per cent were polymorphonuclear leukocytes. The blood leukocyte count returned to normal 10 days after discontinuation of furaltadone or 16 days after ristocetin. A third patient was admitted to our hospital after treatment elsewhere with short courses of chloramphenicol, erythromycin, penicillin, tetracycline, kanamycin, ristocetin, and vancomycin, singly and in combination, all within a two week period, as treatment for staphylococcal empyema. At the time of admission, the urine contained a trace of albumin, and the spun sediment

had 20 to 30 red blood cells/high power microscopic field; the blood urea nitrogen was 19 mg./100 ml. After administration of 1200 mg. of furaltadone per day for two days, large numbers of cellular and hyaline casts appeared in the urine, the blood urea nitrogen had risen to 39 mg./100 ml., and the patient began vomiting. Treatment was continued but the next day the patient had a temperature elevation to 102 F., blood urea nitrogen was reported to be 80 mg./100 ml., and the serum creatinine was 3.3 mg./100 ml. At this time furaltadone was discontinued. One day later the blood urea nitrogen was 33 mg./100 ml. and the serum creatinine was 2.8 mg./100 ml. The temperature returned to normal and the patient stopped vomiting. One week later the blood urea nitrogen was 25 mg./100 ml. and three weeks after treatment it had returned to a normal value of 15 mg./100 ml. The abnormalities in the urine sediment showed a corresponding improvement. Two patients had cutaneous eruption. One of these consisted of hemorrhagic vesicles a few mm. or 1 to 2 cm. in diameter, which appeared on the hands and feet. Many of these ruptured and formed superficial ulcers with little or no inflammation. Another patient developed a florid morbilliform rash, which appeared on the first day of administration of furaltadone and one day after discontinuation of novobiocin, which had been given for six days previously.

DISCUSSION

Substituted furans are a distinct class of antimicrobials. Many are bacteriostatic for both gram-negative and gram-positive microorganisms. Their antibacterial effect is derived principally from the nitration of furan at the 5-position, and is further modified by the substitutions at the 2-position of furaldehyde.^{1,2} A semicarbazone side chain was recognized early as one of the most promising for antibacterial activity against *Staphylococcus aureus*.^{2,5} Furaltadone has these two properties conveyed by the 5 nitrofurfurylideneamino moiety with a carbazide linkage in a substituted oxazolidone. It differs from furazolidone only by the addition of a morpholine group joined through a methyl linkage in the oxazolidone. The 5 nitrofurfuryl structure is common to all the available antibacterial nitrofurans.

The laboratory studies reported here show a significant bacteriostatic and bactericidal activity against staphylococci from furaltadone in concentrations of 4 to 8 µg./ml. There was no relationship to sensitivity or resistance of the strains to antibiotics. The bactericidal action of the drug was significant but not complete at concentrations two- to fourfold greater than the minimal inhibitory concentration, and incubation beyond 24 hours demonstrated some regrowth of the test organism.

The mechanism of the regrowth may be either emergence of resistant clones or degradation of the drug by the culture. In these studies both were shown to occur. We were able consistently to demonstrate loss of antibacterial activity by incubation of furaltadone with cellfree staphylococcal filtrate and a greater loss of activity after incubation with a whole culture of staphylococci.

Resistance was readily induced in vitro by serial passage in subinhibitory concentrations of furaltadone. The pattern was that of an obligatory stepwise mutation usually producing a twofold increase, but occasionally producing a four- to eightfold increase, in the drug resistance of the strain. This pattern closely resembles the in vitro development of resistance of staphylococci to several antibiotics.

Based on these observations it appeared that reliable treatment of staphylococcal infections with furaltadone might be obtained if plasma concentrations at least two to four times the minimal inhibitory concentration for the strain were obtained in vivo and an inhibitory concentration was maintained between doses. Observations of serum levels among patients given 200 mg. every six (or four) hours showed that these goals were not attained. A serum concentration equal to the average sensitivity of the infecting strains was obtained in less than one fourth of the serum specimens and levels two- or fourfold greater than this were rare. In these observations, an inhibitory level was never maintained for six hours after a 200 mg. dose. Indeed, only 12 per cent of samples had any detectable level at that time.

The data might explain the disappointing results obtained with furaltadone in the treatment of patients with staphylococcal infections. A satisfactory clinical and bacteriological response was observed in only one third of the patients, although 75 per cent of those with bacteremia responded satisfactorily. Two of 8 in the latter group, however, had a clinical and bacteriological relapse shortly after discontinuation of treatment courses of 11 and 19 days respectively. Also, of particular importance in evaluating the clinical antistaphylococcal efficacy of the drug was the occurrence of staphylococcal bacteremia in 3 patients during treatment with furaltadone for localized wound infections.

That the nature of the clinical outcome was related to the plasma concentration of furaltadone was shown by the fact that a significantly greater number of serum specimens from patients who had a good response had more than the minimal inhibitory concentration for the infecting strain of *Staphylococcus* than did those from patients who failed to respond, $P = <0.01$. This experience is in keeping with well-established principles of chemotherapy. The failure to attain or maintain inhibitory blood levels is probably attributable to rapid degradation of furaltadone. In vitro studies have shown degradation of the drug by tissues to be as rapid as 18 per cent in 20 minutes.^{3,6} It appears from the differences in the serum levels that there is considerable variation among persons in this regard. Little, if any, furaltadone can be recovered from the stool, but this does not completely rule out the possibility of failure in absorption or destruction of the drug in the gastrointestinal tract.

Although 60 per cent of the patients experienced some form of side reaction during treatment with furaltadone, most of these were minor nausea and/or vomiting or eosinophilia. Neither of these necessitated the discontinuation of treatment in any patient. Of the 2 patients who had a skin eruption, one was very likely due to a previously administered antibiotic and the other related to furaltadone. Of the more serious reactions, one (fever, chills, myalgia, etc.) was unequivocally caused by furaltadone, another (azotemia, urinary casts, vomiting, and fever) was probably caused or certainly aggravated by furaltadone, and the third (agranulocytosis) was most likely coincidental to furaltadone administration. In addition to nausea, vomiting, and eosinophilia, it appears definite that skin eruption is an allergic-type reaction, and some renal irritation may be seen on occasion after treatment with furaltadone. The relationship of furaltadone to bone marrow toxicity is indefinite, as is also the significance of eosinophilia. Although a shortened erythrocyte survival has been observed among persons with primaquine-sensitive erythrocytes, similar to that observed with nitrofurantoin, no hemolytic anemia was recognized among our

patients, one of whom was Negro. The frequency of significant adverse reactions requires further observation, but on the basis of this experience it would appear to be about 8 to 10 per cent.

SUMMARY AND CONCLUSIONS

Furaltadone is a nitrofuran with bacteriostatic and bactericidal activity against staphylococci. Most strains are inhibited in vitro by 4 to 8 $\mu\text{g./ml.}$, and significant numbers of organisms are killed at concentrations two- to fourfold greater than the inhibitory concentrations. Although the drug is quite stable, even in solution, it is altered by tissues and bacteria with consequent loss of antibacterial activity. Resistance to the drug was developed by staphylococci in multiple obligatory steps of two- to fourfold each when strains were subcultured in subinhibitory concentrations.

Dosages of 200 mg. of furaltadone by mouth every four or six hours failed to produce a sustained plasma concentration equal to the minimal in vitro inhibitory concentration for most strains of staphylococci. This concentration, 4 $\mu\text{g./ml.}$, was observed in only 25, 14, and 0 per cent of specimens respectively at two, four, and six hours after a 200 mg. dose. No drug was found in 55, 77, and 88 per cent of serum specimens at two, four, and six hours respectively.

A satisfactory clinical or bacteriological response from furaltadone in the treatment of patients with serious staphylococcal infections occurred in one third of the cases. Some patients showed frank failure manifested by the development of bacteremia during treatment. Relapses after cessation of treatment also occurred. The outcome was significantly but not invariably related to inhibitory concentrations of furaltadone in the plasma during treatment. In no instances was failure related to the development of resistance to furaltadone.

Side effects associated with administration of furaltadone were frequent, but of variable severity. Nausea, vomiting, and slight eosinophilia were most common and least severe. Skin rash, an acute febrile systemic reaction, and renal irritation were observed. Agranulocytosis that occurred during treatment was believed to be coincidental to furaltadone.

Although furaltadone is an effective antistaphylococcal drug in vitro, oral administration does not appear to be a reliable method of treatment because of the variability, low frequency, and poor maintenance of inhibitory concentrations in the blood. Intravenous administration is under study.

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In Vivo Antistaphylococcal Activity of Bisquaternary Diamines

R. J. SCHNITZER, E. GRUNBERG, AND W. F. DELORENZO

Chemotherapy Laboratories, Hoffmann-LaRoche, Inc., Nutley, N. J.

In an earlier publication¹ in which the local antimicrobial activity of triclobisonium chloride* (N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,6-hexanediamine bis[methochloride]) was described, it was also mentioned that this compound exerted a systemic effect in generalized staphylococcal infections of mice. This is an unexpected and unusual property. The experimental evaluation of a great number of chemically related substances, which became available through the extensive chemical work of Goldberg and Teitel of the Roche Research Department, made it possible to circumscribe the occurrence of systemic antistaphylococcal activity in this series of compounds. It could be demonstrated that this property, though not limited to triclobisonium chloride itself, was characteristic of only a small and well-defined group of closely related compounds. Changes in the general chemical constitution of triclobisonium, even comparatively minor ones, resulted in the loss of systemic activity. Examples supporting this statement will be given in this report.

MATERIALS AND METHODS

The following two groups of compounds were studied: Group 1: Homologous bisquaternary alkane diamines derived from β -ionone: (I) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethylethylenediamine bis(methobromide) monohydrate; (II) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,3-propanediamine bis(methobromide); (III) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,4-butanediamine bis(methochloride); (IV) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,5-pentanediamine bis(methobromide); (V) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,6-hexanediamine bis(methochloride) (=triclobisonium chloride); (VI) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,8-octanediamine bis(methobromide); (VII) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,10-decanediamine bis(methobromide); (VIII) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,11-undecanediamine bis(methobromide) hemihydrate; (IX) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,14-tetradecanediamine bis(methobromide) sesquihydrate; Group 2: Compounds related to V (1,6-hexanediamine derivatives): (X) N,N'-Bis[1-methyl-3-(2,6,6-trimethyl-1-cyclohexen-1-yl)propyl]-N,N'-dimethyl-1,6-hexanediamine bis(methobromide) monohydrate; (XI) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,6-hexanediamine bis(methobromide) monohydrate; (XII) N,N'-Dido-

* The trade name of Hoffmann-LaRoche, Inc., for triclobisonium chloride is Triburon.

TABLE I
*Activity of Triclobisonium in the Infection of Mice**

Dose, mg./Kg.	Survival rate: no. survivors/no. treated	
	1 dose	3 doses
50	35/50 (70%)	21/30 (70%)
25	30/50 (60%)	29/40 (73%)
12.5	22/50 (44%)	25/40 (62%)
6.25	6/30 (20%)	11/30 (37%)
Controls	5/60 (8%)	

* Infection: *Staph. aureus* Smith, 100 to 1000 minimum lethal doses intraperitoneally. Treatment by subcutaneous injection.

decyl-N,N'-dimethyl-1,6-hexanediamine bis(methobromide); (XIII) N,N'-Bis(2-cyclohexylethyl)-N,N'-dimethyl-1,6-hexanediamine bis(methobromide) hemihydrate; (XIV) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-1,6-hexanediamine dihydrochloride; (XV) N,N'-Bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-N,N'-dimethyl-1,6-hexanediamine dihydrochloride.

In X, XII, and XIII, the 2,2,6-trimethylcyclohexyl group of V was replaced by 2,6,6-trimethylcyclohexenyl, alkyl, and cyclohexylethyl, respectively. In XI the methyl groups attached to the quaternary nitrogen have been replaced by ethyl groups; XIV and XV are the dihydrochlorides of the secondary and tertiary amines, corresponding to V.

Systemic antistaphylococcal activity was determined in groups of 6 to 10 mice infected intra-abdominally with 0.5 ml. of a 10^{-3} to 10^{-4} diluted suspension of *Staphylococcus aureus* Smith in 5 per cent gastric mucin, corresponding to 100 to 1000 minimum lethal doses. The infection was followed by a single subcutaneous injection of graded dilutions of the substances dissolved in saline. Other groups of mice received the same doses on three successive days. Groups of untreated mice served as controls. Cultures were taken from the organs of animals that succumbed for identification of the recovered organisms. The survivors were observed for 21 days.

The techniques of other experiments presented in connection with the systemic staphylococcal tests were identical with those described earlier.¹ *Staph. aureus* 209 was used in bacteriostatic in vitro tests; owing to its more reliable abscess formation, *Staph. aureus* 503-288 served for the local in vivo experiments at a dose of 0.2 ml. of a $10^{-1.4}$ dilution of an overnight broth culture. Both organisms were known to be highly sensitive to the bisquaternary compounds.¹ The evaluation of the local activity was based on the presence or absence of staphylococcal growth in agar cultures taken from the site of infection 24 hours after local administration of graded dilutions of the substances in water.²

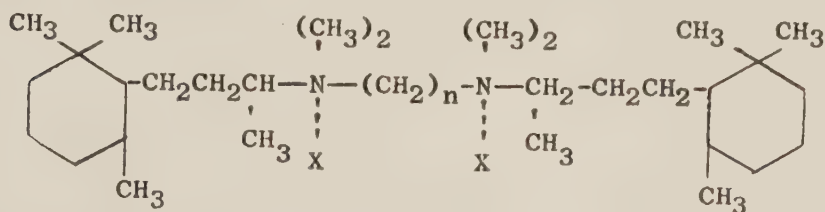
RESULTS

An example of the type of systemic activity in the generalized staphylococcal infection with *Staph. aureus* Smith experiments with triclobisonium (V) is shown in table I. The data of table I indicate that a marked effect on the survival rate

TABLE II

*Homologous Bisquaternary Alkane Diamines in the Group Derived from β -Ionone**

Compound no.	n =	LD ₅₀ , mg./Kg.		Minimal active concentration in vitro, μ g./ml.
		Subcutaneous	Intraperitoneal	
I	2	125.0	37.5	1.56
II	3	175.0	—	1.56
III	4	133.9	18.8	0.78
IV	5	138.7	33.0	0.02
V†	6	153.9	35.0	0.31
VI	8	757.8	—	0.2
VII	10	549.0	—	0.025
VIII	11	297.5	—	0.156
IX	14	175.0	9.4	0.78

* Toxicity for mice; bacteriostatic activity against *Staph. aureus* 209:

X = bromine or chlorine.

† Triclobisonium chloride.

was observed with only a single subcutaneous dose of the drug. Repeated administration was in no case significantly more effective than the single dose. It is not accidental that in no group was the percentage of survivors higher than 70 per cent. This fact is accounted for by the toxicity of the compound, which did not permit the use of higher doses and was also responsible for the death of some animals at the highest dose (50 mg./Kg.) tested. An identical pattern was observed in the experiments with other active members of this series of compounds.

All these compounds that exerted systemic activity belong to group 1, listed previously. Their constitution is recapitulated in table II. Table II presents data concerning the toxicity and in vitro bacteriostatic activity. It is evident that in the series of the bisquaternary alkane diamines, the ethane to the hexane homologues were moderately toxic for mice by the subcutaneous route. The octane and decane compounds were markedly less toxic, but in the higher homologues (VIII, IX), an increase of toxicity was noted. The tetradecane (IX) derivative was also more toxic by the intra-abdominal route, whereas the other members of the series, as far as they have been tested, were tolerated at about the same dose range.

A certain uniformity also governs the high bacteriostatic concentrations of the different members of this group of compounds. With the exception of the pentane (IV) and the decane (VII) homologues, which were of higher activity, all substances showed similar bacteriostatic values in the range of approximately one to two dilution steps, differences that are not considered significant.

These observations were in a general sense confirmed by the experiments in vivo.

Table III presents a comparison of the local effect in the subcutaneous infection by the noninvasive staphylococcal strain with the systemic effect of the

TABLE III

*Local and Systemic Antistaphylococcal Activity of Members of the Series of Homologous Bisquaternary Diamines Derived from β -Ionone**

Compound no.	n =	Antistaphylococcal activity	
		Local, PD ₅₀ , μ g./ml.	Systemic, PD ₅₀ , mg./Kg. subcutaneously
I	2	~12.0	10.6
II	3	5.1	13.4
III	4	10.0	16.9
IV	5	7.5	23.0
V†	6	4.4	16.1
VI	8	2.5	19.7
VII	10	0.5	34.9
VIII	11	1.2	>100
IX	14	~2.0	>25

* Local infection: 0.2 ml. $10^{-1.4}$ *Staph. aureus* 503-288 subcutaneously; systemic infection: 100 to 1000 minimum lethal doses *Staph. aureus* Smith. PD₅₀ = 50 per cent protective dose.

† Triclobisonium chloride.

subcutaneous drug administration in the invasive intra-abdominal infection with *Staph. aureus* Smith.

The values for local activity were expressed as 50 per cent active concentration calculated according to Reed and Muench.³ All compounds exerted a remarkable topical potency, which seems to show a slight increase in the higher members of the series, particularly in the decane and undecane compounds.

Such differences did not occur in the experiments with the generalized infection. The 50 per cent protective doses (calculated according to Reed and Muench from the combined insignificantly different single and repeated treatment groups) were in a comparatively narrow range from the ethane to the decane compound. The average value was 19.2 ± 3 mg./Kg., without a characteristic increase of activity within the series and also without a noticeable optimum. The two highest homologues were not effective systemically.

It might be mentioned that the topical intra-abdominal administration of the compounds in the intra-abdominal infection with *Staph. aureus* Smith reflected the results of the local subcutaneous experiment: All compounds were active in an average range of 18.4 μ g./ml. (minimum 7.4 [VI], maximum 35.4 [VIII]), the higher average value being probably caused by the evaluation on the basis of survival time in this invasive infection.

If one expresses all experimental values of 50 per cent protective doses in mg./Kg., namely, 0.25 mg./Kg. in local subcutaneous tests, 0.92 mg./Kg. in local intra-abdominal tests, and 19.5 mg./Kg. (+ 2 inactive compounds) in systemic tests, the basic difference of the topical and systemic activity becomes even more impressive.

As mentioned before, the occurrence of systemic antistaphylococcal activity is of a limited nature. For instance, this chemotherapeutic effect can be obtained only under conditions of parenteral administration. Probably owing to low intestinal absorption, oral treatment was ineffective. The systemic effect has, moreover, been demonstrated only in *Staph. aureus* Smith. The intravenous infection with *Staph. aureus* Giorgio has not responded in a few experiments.

The influence of the chemical constitution became evident in experiments with the compounds of group 2. Table IV shows the structure, toxicity, and bacteriostatic activity of these compounds.

A comparison with triclobisonium chloride (V), which has been added to table IV as representative of the systemically active substances, shows that essential

TABLE IV
Compounds Related to Triclobisonium Chloride*

Compound no.	Structural formula	LD ₅₀ , mg./Kg. subcutaneously	Minimal active concentration in vitro, µg./ml.
V	$\begin{array}{ccc} (\text{CH}_3)_2 & & (\text{CH}_3)_2 \\ & & \\ \text{R}-\text{N}-(\text{CH}_2)_6-\text{N}-\text{R} \\ & & \\ \text{Br} & & \text{Br} \end{array}$	153.9	0.31
X	$\begin{array}{ccc} (\text{CH}_3)_2 & & (\text{CH}_3)_2 \\ & & \\ \text{R}_1-\text{N}-(\text{CH}_2)_6-\text{N}-\text{R}_1 \\ & & \\ \text{Br} & & \text{Br} \end{array}$	175.0	19.5
XI	$\begin{array}{ccc} (\text{C}_2\text{H}_5)_2 & & (\text{C}_2\text{H}_5)_2 \\ & & \\ \text{R}-\text{N}-(\text{CH}_2)_6-\text{N}-\text{R} \\ & & \\ \text{Br} & & \text{Br} \end{array}$	> 500.0	0.31
XII	$\begin{array}{ccc} (\text{CH}_3)_2 & & (\text{CH}_3)_2 \\ & & \\ \text{CH}_3(\text{CH}_2)_{11}-\text{N}-(\text{CH}_2)_6-\text{N}-(\text{CH}_2)_{11}\text{CH}_3 \\ & & \\ \text{Br} & & \text{Br} \end{array}$	175.0	2.4
XIII	$\begin{array}{ccc} (\text{CH}_3)_2 & & (\text{CH}_3)_2 \\ & & \\ \text{C}_6\text{H}_{11}-\text{CH}_2\text{CH}_2-\text{N}-(\text{CH}_2)_6-\text{N}-\text{CH}_2\text{CH}_2-\text{C}_6\text{H}_{11} \\ & & \\ \text{Br} & & \text{Br} \end{array}$	75.0	950.0
XIV	$\begin{array}{ccc} \text{R}-\text{NH}-(\text{CH}_2)_6-\text{NH}-\text{R} & .2\text{HCl} & \\ & & \\ \text{CH}_3 & & \text{CH}_3 \end{array}$	125.0	0.78
XV	$\begin{array}{ccc} \text{R}-\text{N}-(\text{CH}_2)_6-\text{N}-\text{R} & .2\text{HCl} & \\ & & \\ \text{CH}_3 & & \text{CH}_3 \end{array}$	750.0	3.9
$\text{R} = \begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \quad \\ \text{Cyclohexane ring} \\ \\ \text{CH}_3 \end{array} - \text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)$			
$\text{R}_1 = \begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \quad \\ \text{Cyclohexane ring} \\ \\ \text{CH}_3 \end{array} - \text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)$			

* Toxicity for mice; bacteriostatic activity against *Staph. aureus* 209.

TABLE V

*Local and Systemic Antistaphylococcal Activity of Compounds
Related to Triclobisonium Chloride*

Compound no.	Antistaphylococcal activity	
	Local, PD ₅₀ , µg./ml.	Systemic, PD ₅₀ , mg./Kg. subcutaneously
V	4.4	16.1
X	61.6	>50.0
XI	311.7	>50.0
XII	22.4	>50.0
XIII	>400.0	>25.0
XIV	>400.0	>50.0
XV	296.7	>100.0

Local infection and systemic infection: same as in table III.

differences of toxicity occurred only in compound XI, the diethyl analogue of V, and in compound XV, the tertiary analogue of V. Both were less toxic than the other substances. The bacteriostatic effect of these compounds, with the exception of the cyclohexyl derivative XIII, was still on a comparatively high level.

The corresponding in vivo experiments listed in table V offer, however, an entirely different picture. Local antistaphylococcal activity, if present at all, was on a considerably lower level, with the possible exception of XII, an alkyl analogue of V. The unsaturated compound X and the nonquaternized analogues XIV and XV exhibited very low activity or were inactive at the highest dose tested.

The systemic activity characteristic of the members of group 1 was absent in all instances.

DISCUSSION

It is not possible to discuss any correlations between chemical constitution and systemic activity because at the present time our knowledge of the physical and general biological properties of most of these compounds is too rudimentary to allow more than speculation. However, the experimental evidence of marked systemic activity of the bisquaternary compounds in a staphylococcal infection presented here seems to indicate that they are different from the ionic surface active compounds.

Newton's⁴ recent statement that ionic surface active compounds "are of little chemotherapeutic value" is probably applicable only to monoquaternary compounds, which also, in our experiments, never exerted any systemic antibacterial effect. Different conditions seem to prevail in the case of bisquaternary compounds. We were not able to confirm Kraushaar's⁵ claim that 6,6-dibromo-2,2'-(N,N'-diethylaminoethoxydinaphthyl-(1,1')-N,N'-bis(benzylammonium chloride) influences the infection of mice with the hemolytic *Streptococcus* strain Aronson. However, recent evidence by Hawking and Terry⁶ demonstrated antifilarial activity in the infection of the cotton rat with *Litomosoides carinii* by a series of bis-quinolinium, bis-isoquinolinium, and bis-4-aminocinnolinium compounds.

SUMMARY

In the series of bisquaternary alkane diamines derived from β -ionone, marked chemotherapeutic activity against infections of mice with *Staph. aureus* Smith was observed. The systemic effect was present in the series from the ethane to the decane homologues but was absent in the highest members (undecane and tetradecane) tested. These two substances, however, exerted the bacteriostatic and local in vivo antistaphylococcal effect characteristic for this group of compounds.

Other changes of the chemical constitution, e.g., changes in the ionone part of the structure, its replacement by alkyl groups, and the absence of quaternization, eliminated the systemic chemotherapeutic effect, although in some instances a certain degree of local antistaphylococcal activity was retained.

ACKNOWLEDGMENT

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Erythromycin Propionate in the Treatment of Staphylococcal and Other Infections in Infancy and Childhood

HARRIS D. RILEY, JR., MARY I. ABBOTT, AND WARREN LONG

*Department of Pediatrics and the Children's Memorial Hospital,
University of Oklahoma School of Medicine, Oklahoma City, Okla.*

Since its discovery in 1952 by McGuire and co-workers, erythromycin has become firmly established as a useful and effective antimicrobial agent. Because it is inactivated to varying degrees by gastric secretions, consistent blood levels have not been available with erythromycin base.^{1,2} Several studies concerning the blood levels of erythromycin base in capsules, uncoated and specially coated tablets, and other forms have been presented.²⁻⁴

Several esters of erythromycin have been prepared. Of these esters available, various studies indicate that the propionyl ester of erythromycin has the distinct advantage of producing superior clinical blood levels.^{4,5} This report deals with clinical and laboratory studies of erythromycin propionate.*

METHODS AND MATERIALS

Clinical Studies. Fifty-four infants and children with infections due to different bacterial agents admitted to the Infectious Disease Division of the Children's Memorial Hospital were treated with erythromycin propionate. The disease entities represented in the study group are shown in tables I and II. Thirty-nine of the patients had staphylococcal infections, many of which were severe and life-threatening. In several instances the infection was hospital acquired and due to a strain resistant to the usually employed antimicrobial agents. Ten patients had an associated staphylococcal bacteremia and many patients had multiple clinical expressions of infection concurrently.

Various preparations of erythromycin propionate—capsules, suspension, drops—were utilized at different dosage levels. The dose and duration of the therapy varied with the disease process and are shown in tables I and II. Appropriate pre- and post-treatment cultures and drug susceptibility studies were obtained. Hemoglobin determinations, leukocyte counts, urinalysis, blood urea nitrogen, and other studies as indicated were carried out at regular intervals. All patients were followed carefully from a clinical standpoint for the development of untoward reactions to the drug.

Erythromycin Serum Levels.† Serum samples were obtained for erythromycin blood levels in 16 infants and children. Ten subjects were under treatment with erythromycin propionate. These subjects ranged in age from 3 months to 7 years. All had an infection for which erythromycin was indicated. Erythromycin propionate was administered orally as lauryl sulfate drops in a dose of 20 to 25 mg./

* The trade name of Eli Lilly & Co. for erythromycin propionate is Ilosone.

† Some of these determinations were performed through the cooperation of Dr. R. S. Griffith, Lilly Laboratory for Clinical Research, Indianapolis, Ind.

TABLE I
Summary of 18 Cases

Disease	Age	Causative organism	Type of drug	Dose, mg./lb./day	Durat. therapy, days	Response
Osteomyelitis						
1	16 mo.*	<i>Staphylococcus</i>	Suspension	23.2	46	Satisfactory
2	8 yr.	<i>Staphylococcus</i>	Capsules	24.5	60	Satisfactory
3	2 yr.*	<i>Staphylococcus</i>	Drops	25.0	45	Satisfactory
4	13 mo.	<i>Streptococcus</i>	Drops	35.0	14	Satisfactory
				20.0	26	
Brain abscess						
5	7½ yr.	<i>Staphylococcus</i>	Capsules	40	10	Satisfactory
				16	26	
Subdural empyema						
6	5 mo.*	<i>Staphylococcus</i>	Drops	25.0	9	Satisfactory
Pneumonia						
7	4 yr.†	<i>Staphylococcus</i>	Suspension	22	10	Satisfactory
8	12 days*	<i>Staphylococcus</i>	Drops	20	12	Equivocal
9	9 yr.	<i>Staphylococcus</i>	Suspension	19	15	Satisfactory
10	2 yr.*	<i>Staphylococcus</i>	Suspension	21.3	20	Satisfactory
Bronchiectasis						
11	10 yr.	Mixed flora	Suspension	20	23	Equivocal
Pyelonephritis						
12	6½ yr.	<i>Staphylococcus</i>	Suspension	20	35	Satisfactory
13	10 yr.	<i>A. aerogenes</i>	Suspension	22	14	Unsatisfactory
14	3 yr.	<i>E. coli</i>	Drops	20	10	Equivocal
15	3 yr.	<i>Staphylococcus</i>	Suspension	20	45	Satisfactory
Enteritis						
16	17 mo.*	<i>Staphylococcus</i>	Drops	20	13	Equivocal
17	6 yr.	<i>Staphylococcus</i>	Suspension	25	16	Satisfactory
Parotitis						
18	6 yr.	<i>Staphylococcus</i>	Suspension	20	15	Satisfactory

* Also had staphylococcal bacteremia.

† Also had empyema and bacteremia.

lb./day divided into four equal portions. Blood specimens were obtained before and at two and six hours after the initial dose, the six hour specimen being obtained immediately prior to the second dose. The other 6 subjects were children who had recovered from their original illness or who were hospitalized for non-infectious medical problems. None of the patients was ill at the time of the study. Three patients received erythromycin propionate suspension and 3 patients received the ethyl carbonate form of erythromycin in the suspension form for a total of seven days. All patients received the same dose, 20 mg./lb./day. Other details regarding these groups are shown in table III. Blood was obtained before and at two and six hours after the initial dose on the various days of the study as shown in figure 1.

Serum levels were determined by a modification of the Rammelkamp serial two-fold dilution technique using beta-hemolytic *Streptococcus* C-203 as the indicator organism.

RESULTS AND DISCUSSION

Erythromycin Serum Levels. The average serum levels of antistreptococcal activity after the oral administration of erythromycin propionate in the lauryl sulfate form are presented in figure 1. This represents the mean response of 10 infants and children under treatment with this form of erythromycin propionate. Blood was obtained before administration of the drug, at two and six hours after the initial

TABLE II
Summary of 36 Cases

Disease	Causative organism	No. patients	Dosage range, mg./lb./day	Av. durat. therapy, days	Result
Soft-tissue infections					
Wound infections	<i>Staphylococcus</i>	5	20-35	15	Satisfactory 4 Unsatisfactory 1
Pyoderma and skin abscesses	<i>Staphylococcus</i>	19*	20-32	15	Satisfactory 16 Unsatisfactory 2
	<i>Streptococcus</i>	3	20	10	Equivocal 1 Satisfactory 2
Otitis media	<i>Staphylococcus</i>	1	20	20	Equivocal 1 Satisfactory 1
	<i>Streptococcus</i>	1	18	16	Satisfactory 1
	<i>H. influenzae</i>	2	24	12	Unsatisfactory 2
Pharyngotonsillitis	<i>Streptococcus</i>	5	20-28	10	Satisfactory 3 Unsatisfactory 2

* Three patients had staphylococcal bacteremia.

dose. As can be seen in figure 1, within two hours a prompt rise in the serum concentration had occurred. By six hours the mean level was greater than 1:256. This level was attained by all except 2 subjects and in these the level was only slightly lower. These levels are significantly higher than the levels at two and six hours obtained with erythromycin propionate in a gelatin capsule.⁴

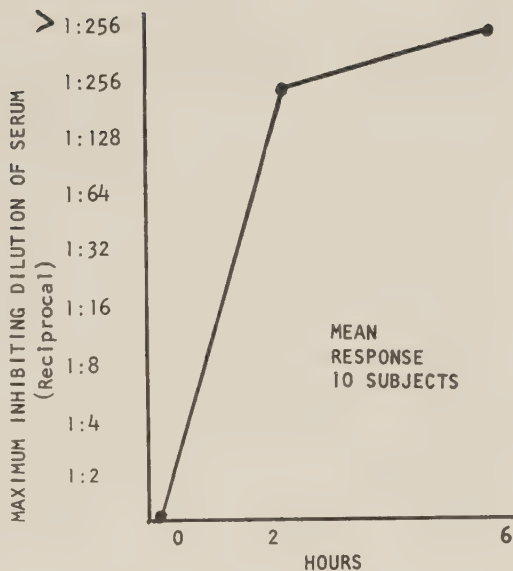
In figure 2 are presented the comparative serum levels in 3 subjects who received erythromycin propionate as the suspension and in 3 subjects who were given erythromycin as ethyl carbonate in suspension. All subjects had recovered from their original illness or were hospitalized for non-infectious medical problems. None of these was ill at the time of the study. Each of the 6 subjects received the same dose (20 mg./lb./day divided into four equal doses) of one of the two erythromycin forms for seven days. It can be seen that the serum concentrations following the propionyl ester were characterized by a more prompt rise and more sustained levels than occurred with the ethyl carbonate form. Figure 2 also shows that the concentration 12 and 24 hours after stopping the drug was consistently higher with the propionate than with the ethyl carbonate form.

Clinical Results. The pertinent details concerning 54 infants and children treated with erythromycin propionate are shown in tables I and II. Several of the patients had severe staphylococcal infections with hospital-acquired, drug-resistant strains

TABLE III
Details of Patient Population in Whom Comparison of Blood Levels Following Administration of Erythromycin as Ethyl Carbonate and as Propionyl Ester Was Carried Out

Patient	Diagnosis	Age, yr.	Weight, lb.	Type of drug in suspension	Dose, mg./lb./day
1	Diabetes	6	41	Propionate	20
2	Rheumatic fever	12	115	Propionate	20
3	Convulsive disorder	2½	30	Propionate	20
4	Convulsive disorder	5½	50	Ethyl carbonate	20
5	Post-poliomyelitis	3	32	Ethyl carbonate	20
6	Hyperthyroidism	10	61	Ethyl carbonate	20

FIG. 1. Serum inhibiting levels for the test organism in 3 subjects receiving propionyl erythromycin in suspension form and in 3 subjects receiving ethyl carbonate form of erythromycin as suspension. The dose in both groups of subjects was 20 mg./lb./day.

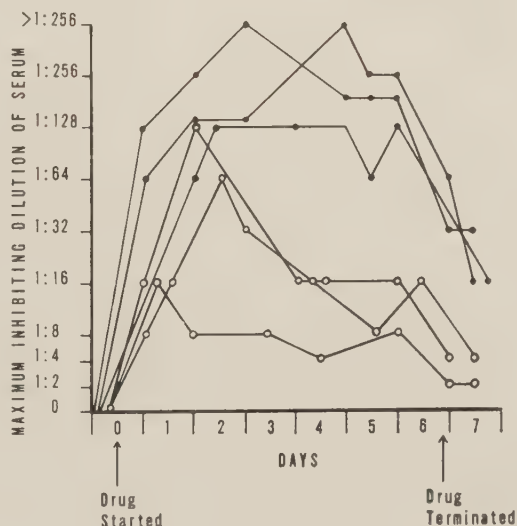


and had been unsuccessfully treated with other antibacterial agents prior to erythromycin propionate therapy. Of the 39 patients with staphylococcal infections, 10 had bacteremia. Many of the patients had multiple clinical expressions of infection concurrently.

Ten patients had infections due to group A streptococci but with the exception of a child with streptococcal osteomyelitis and 1 patient with a severe cellulitis and abscess formation, they were not as severely ill as were those with staphylococcal infections.

Various preparations of the drug—capsules, suspension, and lauryl sulfate drops—were utilized at different dosage levels. The duration of therapy varied with the nature of the disease process. Although no blood level studies were carried out with the capsule form of erythromycin propionate, it was our clinical impression

FIG. 2. Inhibiting serum levels in 10 pediatric subjects after ingestion of one dose of propionyl erythromycin as lauryl sulfate drops 20 to 25 mg./lb. ●—●, erythromycin propionate; ○—○, erythromycin ethyl carbonate.



that patients obtained a more rapid therapeutic response to erythromycin propionate when used as a suspension or drops than with capsules.

Staphylococcal Infections. Three patients with staphylococcal osteomyelitis responded satisfactorily to erythromycin propionate. One patient, a 2 year old white boy, was admitted with osteomyelitis of the tibia with a draining subcutaneous sinus. On erythromycin propionate cultures of the blood and sinus drainage promptly became sterile. Radiologically, there was satisfactory healing of the lesion. An 8 year old boy with osteomyelitis of the pubis demonstrated satisfactory roentgenographic healing after 60 days of therapy.

A seven and one-half year old boy with brain abscess following acute mastoiditis was treated for 36 days with erythromycin propionate. There was a very satisfactory clinical and bacteriological response. A 5 month old infant with a staphylococcal empyema of the subdural space also exhibited an excellent response to erythromycin propionate.

Four patients with proved staphylococcal pneumonia responded adequately to the drug. In 1 patient, a 12 day old infant, there was prompt eradication of staphylococci from the blood stream, but the child continued to have respiratory distress. In all probability, the infection had been satisfactorily controlled, but pleural complications caused us to consider the final result in this patient equivocal.

Of the other patients with staphylococcal disease, 2 with postoperative enteritis, 1 with pyelonephritis, and a 6 year old girl with suppurative parotitis all obtained satisfactory responses to propionyl erythromycin.

Twenty-four patients with soft-tissue infections due to hemolytic *Staphylococcus aureus* were treated with erythromycin propionate. Five of these were wound infections and 19, pyoderma, in most cases quite extensive and with skin abscesses. As shown in table II, 20 of these patients were considered to have a satisfactory, 3 unsatisfactory, and 1 an equivocal response to therapy. Those classified as unsatisfactory and equivocal were, in all probability, due to the advanced stage of the disease when the patients were seen or a delay in instituting surgical drainage.

Although the clinical and bacteriological response in the 10 patients with staphylococcal bacteremia was not so dramatic as might have been expected with an antibacterial agent given parenterally, the final results in all such cases were satisfactory.

Streptococcal Infections. A 13 month old infant with streptococcal osteomyelitis of the humerus exhibited a satisfactory clinical, bacteriological, and roentgenographic response to 40 days of erythromycin propionate. Three patients with extensive pyoderma and skin abscesses were treated with erythromycin propionate with a satisfactory result in 2 instances and equivocal in 1, probably due to delay in initiating the medication in this particular patient. Three of 5 cases of streptococcal tonsillitis responded as did one case of streptococcal otitis media.

Infections due to Other Organisms. The response of a 10 year old boy with bronchiectasis due to a mixed flora was equivocal in regard to clearing of infection. There was no improvement in a patient with *Aerobacter aerogenes* pyelonephritis to the drug and the response of another patient with pyelonephritis due to *Escherichia coli* was equivocal. Three infants with acute suppurative otitis media due to *Hemophilus influenzae* did not respond.

Drug Susceptibility Studies. Of the strains of staphylococci and streptococci re-

cently isolated and subjected to in vitro susceptibility testing, the great majority were susceptible to erythromycin. The staphylococcal strain from a patient with osteomyelitis became resistant in vitro to erythromycin after 30 days of therapy with erythromycin propionate.

Side Effects. All patients tolerated the medication well. The absence of nausea and vomiting was quite striking, even in patients who received the medication for as long as two months. No skin rashes or other untoward effects were noted in the series.

Dose. On the basis of these studies of blood levels of erythromycin and clinical evaluation in various infections, it is felt that a dose of 20 to 25 mg./lb./day is a satisfactory dose for the majority of erythromycin-sensitive infections of infancy and childhood. In our experience with more severe infections, no untoward effects were encountered when doses as high as twice the dose considered satisfactory were administered for several days. Propionyl erythromycin both in the suspension and lauryl sulfate forms was palatable and readily accepted by both infants and young children.

SUMMARY

Erythromycin blood levels in 10 infants and children under treatment with erythromycin propionate 20 to 25 mg./lb./day as the lauryl sulfate form were characterized by a prompt rise in two hours and maintenance at high levels for at least six hours after an initial dose. Blood levels in 3 other patients receiving 20 mg./lb./day were maintained at high levels during a seven day study period. When both were administered in suspension form in a dose of 20 mg./lb./day, propionyl erythromycin produced consistently higher and more prolonged serum concentrations than did the ethyl carbonate form of erythromycin.

Erythromycin propionate was employed therapeutically in the treatment of 54 infants and children. Of the 39 with staphylococcal infections, many of which were clinically severe and due to hospital-acquired strains resistant to other antibiotics, a satisfactory response was obtained in 33. Seven of 10 patients with streptococcal infections responded satisfactorily. Too few cases due to other bacterial organisms were treated to afford any conclusions.

The drug was well accepted and there were no untoward side effects.

ACKNOWLEDGMENT

The valuable assistance of the house officers on the Pediatric Service in studying these cases is appreciated.

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The Use of Vancomycin in Pediatrics

ROBERT L. SPEARS AND RICHARD KOCH

*Department of Pediatrics, University of Southern California School of Medicine,
and the Childrens Hospital of Los Angeles, Los Angeles, Calif.*

Vancomycin* is a new antimicrobial agent isolated from *Streptomyces orientalis*. This antibiotic has excellent bactericidal activity, chiefly against gram-positive bacteria,¹⁻³ and its principal therapeutic usage has been for treating patients with serious staphylococcal infections. Since this antibiotic has only been administered intravenously, its use in infants and children has been limited, due to the difficulty in maintaining continuous intravenous administration. The purpose of this report is to present preliminary data on methods of administration, serum concentrations, clinical effectiveness, and toxicity in 53 children admitted to the Los Angeles Childrens Hospital.

METHODS AND MATERIAL

For serum determinations, vancomycin was administered to 16 patients intravenously and intramuscularly to 14 others. Vancomycin was provided by the manufacturer in 10 ml. vials, which contained 0.5 Gm. These were diluted with sterile water to a concentration of 50 mg./ml. and then further diluted with 20 ml. of either glucose or saline solutions, which were then administered intravenously over a period of three to five minutes. For intramuscular use, the intravenous preparation was diluted to a concentration of 100 mg./ml. of diluent and mixed with 1 mg. of hydrocortisone.

For serum and cerebrospinal fluid level determinations, the standard twofold tube dilution method was utilized, using *Streptococcus pyogenes* ATCC 8668 as the test organism. Patients utilized for serum data were children without metabolic or renal disease. Blood samples were collected by venipuncture, allowed to clot, centrifuged, and the serum stored at -17 C. until sufficient numbers were collected for determination of vancomycin content.

Only seriously ill patients suspected of having staphylococcal infections were selected for vancomycin therapy. All patients receiving the antibiotic were utilized for gathering toxicity data. The dosage used was 25 to 60 mg./Kg./day, except for 5 patients who received 100 mg./Kg./day or more.

RESULTS

Methods of Administration. Several methods of administration were attempted because of the high incidence of severe thrombophlebitis, associated with the intravenous administration of vancomycin. Continuous intravenous drip proved most satisfactory. Even so, 25 per cent treated in this manner developed thrombophlebitis, which necessitated changing intravenous sites every 24 to 36 hours. The concentration of vancomycin administered was 1 mg./ml. of diluent. Nearly all of

This study was supported by a grant from Eli Lilly & Co.

* The trade name of Eli Lilly & Co. for vancomycin is Vancocin.

the patients receiving direct intravenous injection experienced flushing, itching of the skin, and a general sense of discomfort. One 11 year old boy developed urticaria and wheezing respirations while receiving a rapid intravenous drip containing 20 mg./Kg. Another 10 month old infant died unexpectedly following administration of 60 mg./Kg. by the rapid drip technique administered during a 30 minute period. Ten minutes after discontinuance of the antibiotic she became cyanotic, developed cardiorespiratory arrest, and died, in spite of artificial respiration, cardiac massage, and adrenalin. Autopsy revealed an overwhelming bronchopneumonia. Blood, nasopharyngeal, and tracheal cultures obtained prior to and at the time of autopsy for bacterial growth were sterile. Serum concentration at the time of death was 13 μ g. of vancomycin. It is not known whether her death was directly related to the antibiotic therapy or due to the extensive disease process itself, which, in retrospect, was presumed to be viral in etiology.

In an effort to minimize thrombophlebitis during intravenous therapy, hydrocortisone, in a concentration of 1 mg./100 mg. of vancomycin was added to the intravenous fluids administered to 2 patients; however, this did not appear to be of any benefit. Heparin, 10 mg./500 ml. of diluent, was also tried without success in 2 patients. Intramuscular trypsin was tried in dosage of 1 ml. daily for three days in 1 patient, also without apparent benefit.

The intramuscular administration of vancomycin mixed with hydrocortisone was also evaluated. The dosage utilized was 40 mg./Kg. body weight/day of vancomycin. The combination of vancomycin and hydrocortisone was administered to 14 normal children for serum level determinations and to 6 other patients for therapy of acute infections. This route of administration was found to be most useful for treating small infants in whom intravenous therapy was technically quite difficult. Older children complained of persistent pain following injection; however, no other reactions were noted in the 20 patients who received vancomycin by the intramuscular route, although 2 infants received it for 12 and 16 days, respectively.

Serum Concentrations. Vancomycin was administered as a single intravenous injection of 20 mg./Kg. to 16 patients. Thirty samples of serum were then collected at times varying from 1 to 12 hours after the initial injection of vancomycin. Figure 1 shows that there is a gradual fall in serum concentrations from a peak value of

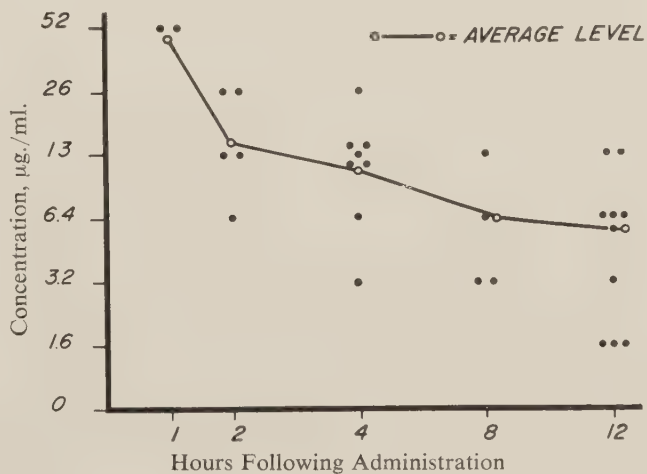


FIG. 1. Vancomycin serum content following a single intravenous injection of 20 mg./Kg. of body weight.

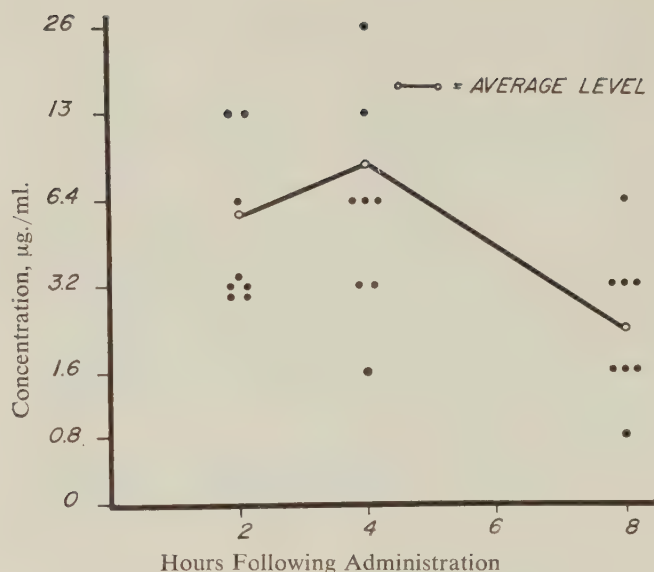


FIG. 2. Vancomycin serum content following a single intramuscular injection of 13 mg./Kg. of body weight.

52 µg./ml. at one hour to a range of 1.6 to 13 µg./ml. 12 hours after administration. There was found to be no difference in the average values obtained from 5 patients under the age of 2 weeks as compared to the group as a whole.

Vancomycin was given by intramuscular injection to 14 patients in a dose of 13.3 mg./Kg. A smaller dosage was chosen for intramuscular therapy because the manufacturer does not advocate this route of administration. Twenty-four serum samples were collected at varying times after the initial injection. The results of these serum determinations are shown in figure 2. It is of interest that the peak concentration was four hours after the antibiotic was administered, and therapeutic levels were maintained for at least eight hours following injection.

Cerebrospinal Fluid Concentrations. Seven cerebrospinal fluid samples were obtained from subjects at various intervals of time, ranging from 1 to 12 hours after the initial intravenous or intramuscular injection of vancomycin in the previously mentioned dosage. In all cases the cerebrospinal fluid contained a level of vancomycin activity of less than 0.8 mg./ml., indicating that vancomycin apparently does not cross into the cerebrospinal fluid in detectable amounts in patients without central nervous system infection.

Clinical Effectiveness. Vancomycin was given as the therapeutic antibiotic agent to 23 patients. Table I summarizes the data collected on these patients. Their ages ranged from 1 month to 18 years. The dosage of vancomycin given varied from 40 to 180 mg./Kg./day; the most frequently used dose was either 40 or 60 mg./Kg./day. The intravenous route of administration was utilized exclusively in 17 patients; the intramuscular route in 3 and a combination of both in 3 others. All patients were seriously ill, and many of them had other complicating illnesses, such as leukemia and congenital heart disease. *Staphylococcus aureus*, coagulase positive, was isolated by bacteriological culture in 20 of the 23 cases. In 1 case, beta-hemolytic *Streptococcus* was isolated, *Hemophilus influenza* type B in 1, and no organism was recovered in 1. The duration of therapy varied from a few hours to 28 days of continuous therapy. It was difficult to evaluate the results of therapy due to

many complicating factors in these patients. An attempt was made to classify the therapeutic results as follows: cured, improved, unimproved, and died. In the 5 fatal cases, death was due to the original disease process and not the infectious process itself in 3. (See table I.) For this reason, no valid figures on mortality rate can be given. Of the treated patients, 12 (52 per cent) were considered cured, 2 (9 per cent) improved, 4 (18 per cent) unimproved, and 5 (22 per cent) died. Only 2 of these patients received other antibiotics concurrently with vancomycin, and the majority had experienced therapeutic failures with other antibiotics prior to trial with vancomycin.

TOXICITY

Patients who received only a single injection of vancomycin for serum level determination experienced minimal side effects. The most commonly observed symptom

TABLE I
Collected Data on 23 Patients Treated with Vancomycin

Patient no.	Age, yr.	Sex	Dose, mg./Kg./day	Diagnosis	Organism	Length of therapy, days	Toxicity	Result
1	3 mo.	M	40	Pneumonia and empyema	<i>Staphylococcus</i>	13		Cured
2	8	M	60	Abscess	<i>Staphylococcus</i>	4		Cured
3	10	M	40	Subacute bacterial endocarditis	<i>Staphylococcus</i>	9	Phlebitis	Improved
4	13	M	60	Osteomyelitis	<i>Staphylococcus</i>	3	Rash	Unimproved
5	2	F	40	Pneumonia	<i>Staphylococcus</i>	17		Cured
6	3	F	100	Sepsis	<i>Staphylococcus</i>	10		Cured
7	4	F	100	Fibrocystic with bronchopneumonia	<i>Staphylococcus</i>	8		Died
8	9	M	60	Sepsis and osteomyelitis	<i>Staphylococcus</i>	16	Phlebitis	Cured
9	3 mo.	M	100	Bronchopneumonia	<i>Staphylococcus</i>	14		Cured
10*	1 mo.	F	40	Pneumonia and empyema	<i>Staphylococcus</i>	20		Died
11*	4	M	100	Bronchopneumonia	<i>Staphylococcus</i>	13		Cured
12	14	F	40	Sepsis	<i>Streptococcus</i>	4		Cured
13	6 wk.	F	60	Sepsis	<i>Staphylococcus</i>	5		Cured
14	1	F	40	Abscess and cellulitis	<i>Staphylococcus</i>	6	Renal	Cured
15	6	M	100	Leukemia and sepsis	<i>Staphylococcus</i>	7	Phlebitis	Died
16	3	F	60	Pneumonia	<i>Staphylococcus</i>	3		Cured
17	4	M	60	Multiple abscesses	<i>Staphylococcus</i>	2	Rash	Unimproved
18	5	M	40	Pneumonia	Type B <i>H. influenzae</i>	3		Unimproved
19	10	M	40	Uremia and subacute bacterial endocarditis	<i>Staphylococcus</i>	4		Died
20	12	F	25	Subacute bacterial endocarditis	<i>Staphylococcus</i>	15		Unimproved
21	1	M	180	Pneumonia	—	<1	Sudden death	Died
22	18	M	40	Subacute bacterial endocarditis	<i>Staphylococcus</i>	28	Phlebitis	Cured
23	4	M	40	Pneumonia and empyema	<i>Staphylococcus</i>	7	Phlebitis, rash	Improved

* Received other concurrent antibiotic therapy.

(90 per cent) was transient flushing of the body lasting 5 to 15 minutes. Many of the older patients complained of transient itching, especially on the posterior aspect of the neck. No hives, sterile abscesses, rashes, or thrombophlebitis were noted in this group of patients; however, 1 patient who received a single intramuscular injection developed erythema and tenderness at the site of injection, lasting two days.

The chief difficulty noted in patients receiving vancomycin therapeutically was thrombophlebitis at the site of intravenous administration, the complication being present in 5 of 20 patients (25 per cent) who received vancomycin intravenously. Subsequently this complication was diminished by frequent changing of the site of intravenous administration. Three of the patients developed maculopapular rash, which required discontinuance of the drug. One patient developed albuminuria and microscopic hematuria, which cleared spontaneously with discontinuance of the antibiotic. No concomitant elevation of blood urea nitrogen was noted. Possibly the most serious reaction was the sudden death of the patient who died following administration of vancomycin by rapid intravenous drip. The low serum concentration of 13 µg. documented premortem suggests that the cause of death probably was the overwhelming disease process itself, although a hypersensitivity reaction cannot be ruled out.

DISCUSSION

The development of severe thrombophlebitis (25 per cent) due to intravenous administration of vancomycin makes this antibiotic technically difficult to use for treating children. Fortunately, this complication in children is not as serious as it is in adults and can be prevented by changing the intravenous site every 24 to 36 hours. Another disturbing feature of this problem, which may worry the clinician unnecessarily, is that fever frequently accompanies the development of thrombophlebitis; however, this subsides promptly with the resolution of the thrombophlebitis.

Rapid intravenous injection of this antibiotic is contraindicated at the present state of our knowledge until more data are available. Continuous intravenous infusion is the preferred route of administration.

Due to persistent pain experienced by older children, the intramuscular route is not recommended. In premature and small infants, as well as in seriously ill children in whom intravenous administration is technically difficult, the intramuscular route can be used safely and effectively.

Kirby and Divelbiss² found that 51 of 63 strains of staphylococci isolated from various types of infections were inhibited in vitro by 2 µg./ml. of vancomycin, the remaining 12 requiring concentrations up to 3 µg./ml. Group A streptococci and pneumococci were found to be two to four times more sensitive than the staphylococci tested. Another investigator, using *Micrococcus pyogenes* var. *aureus* 209 P, found that 1.56 µg./ml. destroyed 99 per cent of the bacteria in 24 hours of incubation.³ Geraci et al⁴ reported that vancomycin in a concentration of 2.5 µg./ml. completely inhibited 110 of 112 strains of *M. pyogenes* tested in vitro. It is readily apparent from the data reported herein that a dosage of 40 mg./Kg./day intravenously or intramuscularly easily provides adequate therapeutic serum levels of van-

comycin in children. Due to the incidence of side reactions and difficulty of administration, larger dosage would seem unwarranted.

Vancomycin is an effective antibiotic for treating serious staphylococcal infections.⁵ It is our impression that it may be utilized alone, except in such cases where the antibiotic must cross tissue barriers, such as in meningitis and empyema pockets. In such cases other antibiotics should be administered concurrently with vancomycin, if the latter is selected for therapy.

The high incidence of toxicity (40 per cent) experienced by our patients treated with vancomycin may be related to the higher dosage utilized, due to our unfamiliarity with this antibiotic. On the other hand, one cannot overlook the difficulties associated with its administration, and for this reason it should be utilized only for treating seriously ill, hospitalized patients who can be kept under close medical surveillance.

SUMMARY

The purpose of this paper has been to present preliminary data on the methods of administration, serum concentrations, clinical effectiveness, and toxicity of vancomycin in a group of 53 patients studied at the Los Angeles Childrens Hospital. Based upon the data gathered in this report, the recommended dosage should be 40 mg./Kg./day administered intravenously. Continuous infusion technique is the preferred route of administration.

Vancomycin does not diffuse into the cerebrospinal fluid in detectable amounts. Vancomycin was given as a therapeutic agent to 23 patients seriously ill with various staphylococcal infections. Sixty per cent of these responded with cure or improvement, 18 per cent were unimproved, and 20 per cent died. It is our impression that vancomycin is an excellent antibiotic for the treatment of seriously ill patients with staphylococcal infections that have failed to respond to other antibiotics.

Although the incidence of side reactions was high, no severe toxic manifestations were noted, with the possible exception of an infant who died shortly after receiving a rapid intravenous drip of vancomycin.

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The Use of Tetracycline-Oleandomycin Combination in the Acute Pneumonopathies of Infancy, with Particular Consideration of Staphylococcal Empyema

RODOLFO BURDACH, HAYDEE SEPULVEDA, CARMEN AGUILO, AND
SANTIAGO RUBIO

Department of Pediatrics, Manuel Arrirán Hospital, Santiago, Chile

There is a valid reason for the testing of new antibiotics in the treatment of acute pneumonopathies in our hospital, inasmuch as these diseases constitute one of the most important causes of morbidity and are of a particularly severe character in infancy.

According to the records of our Department of Pediatrics in the Nursing Infants' Section, the mortality of the acute pneumonopathies had remained at about 10 per cent in the course of the past years.¹⁴ In the Emergency Section of the same hospital, the corresponding figure rose as high as 41.9 per cent in 1958.¹⁹ The explanation of this striking difference is that this particular section houses children who are in an extremely serious condition and who are then moved to the various sections of the hospital once they are past the critical stage.

The rise in the incidence of staphylococcal pneumonopathies has been obvious in the course of the past three years, as is demonstrated by the large number of pleural empyemas of this etiology that we have observed during the same period.¹⁵⁻¹⁷

Staphylococcal pneumonopathies are known to be more serious in character in view of their hyperacute clinical forms, high incidence of pleural complications, and the increase in resistance of pathogenic microorganisms to the customary antibiotics.^{1, 3, 5-10, 12, 13, 16-18, 20, 21}

In Chile, moreover, malnutrition,¹³ so frequent in children of the poorer classes, is a factor that makes these clinical findings particularly serious.

Accordingly, the tetracycline-oleandomycin combination was utilized to treat a group of children with acute pneumonopathy, who were hospitalized.

MATERIAL AND METHOD

Case material comprised 49 cases, 25 of them complicated by pleural empyema. These are analyzed separately.

Uncomplicated Acute Pneumonopathies (Table I). This group consisted of 24 patients; their ages varied from 2 months to 3 years, and 17 of them were less than a year old.

The nutritional state of 18 children was satisfactory, and that of 5 was deficient (less than 80 per cent of the ideal weight). A marked general involvement that justified hospitalization was present in all of them.

Duration of the disease prior to hospitalization varied from one to eight days, with an average of 2.8 days. During this period, 9 children had received some antibiotic, but every child was admitted in a serious condition, as pointed out previously.

The diagnosis of pneumonopathy was confirmed radiologically in 23 of the patients. Pathology was bilateral in 10 cases.

Treatment consisted of oral administration of tetracycline-oleandomycin combination; in certain serious cases, intravenous administration was also employed at the initiation of therapy. General adjuvant measures included humidification, oxygen therapy, parenteral hydration, etc.

The oral tetracycline-oleandomycin combination was used in syrup form, in daily dosages of 0.4 to 1.0 Gm. given in four fractional doses at six hour intervals. The dose per Kg. of body weight per 24 hours varied between 50 and 100 mg.

RESULTS. Clinical improvement was observed in 18 children on the day after initiation of treatment. Fever vanished in one to three days in 20 of these patients. Signs of pulmonary condensation subsided in a few days except in 2 cases in which the disease did not have a prolonged course. Bronchial symptoms persisted for a longer period. A radiological control examination was performed in 17 children, revealing a normal condition in all of them.

Pleural Empyema (Table II). In this group of children, the tetracycline-oleandomycin combination was used alone in 11 patients, whereas in the other 14 it was applied along with other general or intrapleural antibiotics.

We will discuss this group as a whole, as there were no appreciable differences in the development of the disease that could be attributed to the effect of the additional antibiotic therapy.

The ages of the 25 children in this group varied from 15 days to more than 3 years, and 12 of them were less than a year old.

The nutritional state of 19 children was satisfactory, whereas it was regarded as deficient in the other 6 (less than 80 per cent of the ideal weight).

The condition was markedly severe in 24 children; in only 1 case was the general involvement less manifest.

Duration of disease prior to the initiation of treatment with the tetracycline-oleandomycin combination varied from 2 to 32 days. These children, as indicated in table II, were treated with the tetracycline-oleandomycin combination jointly with other antibiotics, since the previously administered therapy could not control the patient's serious infectious state.

The diagnosis of pleural empyema was based on the clinical picture and the radiological examination, and it was confirmed by thoracentesis.

Radiology revealed effusion in 9 children and pyopneumothorax in the other 16. In 2 patients, moreover, a picture of pneumonic consolidation was observed on the opposite side.

All these empyemas were classified as staphylococcic, based on clinical characteristics, radiological features, histological studies in cases of death or surgery, as well as on the finding of staphylococci in the pleural exudate (10 cases).

The tetracycline-oleandomycin combination was administered orally, in syrup form, in a daily dosage of 0.5 to 1.2 Gm., in fractional doses given at six hour intervals. The total dosage of the therapy varied from 4.4 to 53 Gm., with an average of 25 Gm. The combination drug was administered for a period that varied from 11 to 53 days, with an average of 29.8 days. Two patients died after receiving the antibiotic for only two and four days, respectively; consequently these were not included in the fore-mentioned averages (cases 1 and 4).

TABLE I
Twenty-four Cases of Uncomplicated

No.	Age	Weight, Gm.	% ideal weight	Days of disease	General condition		Cyanosis	Pulmonary roentgenograms	
					Severe	Mod. severe		Left	Right
1	2 mo.	4.600	110	2	+		+	+	+
2	3 mo.	4.000	80	3	+		+	+	+
3	3 mo.	5.000	100	3	+		+		+
4	3 mo.	2.300	48	1		+		+	+
5	3 mo.	4.500	90	2		+			+
6	4 mo.	5.000	90	2	+		+	+	+
7	4 mo.	4.000	70	3	+				+
8	4 mo.	5.400	98	4	+		+		+
9	4 mo.	5.800	105	2	+				+
10	4 mo.	5.200	90	8	+			+	+
11	5 mo.	5.200	85	3		+		+	
12	5 mo.	4.000	70	2	+		+		+
13	6 mo.	8.900	120	1	+				
14	6 mo.	6.000	90	5	+			+	+
15	8 mo.	7.100	90	3	+				+
16	9 mo.	10.250	120	3		+		+	+
17	10 mo.	7.100	85	1	+				+
18	13 mo.	10.000	100	3		+			+
19	14 mo.	9.200	100	4	+		+	+	+
20	14 mo.	8.900	100	3		+			+
21	15 mo.	9.200	90	3	+			+	
22	15 mo.	9.800	100	1	+		+	+	
23	24 mo.	8.400	75	5	+			+	+
24	3 yr.	10.400	75	2	+		+	+	+

In certain patients treated this year, in conformity with a previously established plan, the tetracycline-oleandomycin combination was given initially in phlebotomy, in doses of 250 to 500 mg. diluted in 250 to 500 ml. of glucose or glucosaline solution. This intravenous dose was repeated on the second or third day in some cases.

For intrapleural administration, the tetracycline-oleandomycin combination was used in a dosage of 100 Gm. daily, in 11 children; chloramphenicol succinate, in a dosage of 250 to 500 mg. daily, in 13; sodium penicillin, in a dosage of 500,000 units, in 1 child. Intrapleural therapy was continued in accordance with duration of pleural drainage or the number of thoracenteses. The evacuation of pleural exudate was performed by thoracentesis in 5 children and by thoracotomy with closed drainage in 20.

RESULTS. As for the progress observed in these patients, clinical improvement was noted within a period that varied from 3 to 45 days, the average being 14.5 days. Fever, a constant symptom, disappeared in the majority of cases along with the other signs of toxemia.

Eleven of the 25 children showed complete improvement within a period of 11 to 130 days. Minimal pleural opacity was noted in 6 patients during a period of observation that ranged from 26 to 90 days. In 2 patients, a small marginal pneumothorax was revealed on the thirty-second and sixtieth days, respectively. Pulmonary surgery was performed in 3 patients. One of them underwent pneumo-

TABLE I

Acute Pneumonopathies

Tetracycline-oleandomycin			Evolution			Duration, days		Normal roentgenogram, days
Intrave- nous	Daily dose, oral	Total dose	Therapy, days	Improve- ment, days	Fever, days	Consolida- tion	Bronchial signs	
0.25	0.5	3.5	7	2	2	3	8	8
	0.4	2.8	7	2	1	3	5	
0.25	0.5	6.5	13	2	2	3	5	8
	0.25	4	8	2	1	2	4	
	0.5	4	8	3	6	2	5	
	0.5	3	6	2	1	2	6	7
0.25	0.5	3.5	7	2	1	5	4	9
0.25	0.5	5.5	11	2	2	2	6	9
0.25	0.5	10	20	5	14	13	18	25
	0.4	2.25	6	2	1	2	7	6
0.25	0.4	1.75	6	2	1	2		5
0.25	0.4	4	8	2	2	4	13	16
0.25	0.5	5.7	11	2	3	4	9	
0.25	0.5	3.5	7	2	3	5	9	12
	0.5	5.5	11	3	3	3	3	6
	0.5	5	10	2	3	5	5	6
	0.5	5	10	2	1	2	12	15
	0.5	3.5	7	3	4	2	4	8
0.5	1	5	5	2	2	3	4	
0.5	0.5	4	8	3	1	2	9	10
	0.5	2	4	2	1	3	6	7
	1	6	6	2	3	3	7	
	0.5	4	8	2	3	4	9	
0.5	1	12	12	4	6	4	16	17

nectomy (case 8), decortication was done in the second (case 9), and resection of a pneumatocele was performed in the third (case 21). The subsequent course of these patients was satisfactory.

Three patients died, 2 of them in the acute phase of infection (cases 1 and 4) and the third of surgical accident (case 24).

COMMENT

In our hospital environment, acute pneumonopathies in the child are serious conditions^{13-17, 19} that justify the trial of new antibiotics.

Among the factors that influence the prognosis of this disease, a prominent part is played by previous nutritional state,¹³ which was deficient in 25 per cent of our patients. Another important factor to be considered is the age of the patient, since a higher mortality occurs in the lower age brackets;^{7, 10, 15-17} in our case material 61 per cent of the children were less than a year old.

Owing to the present increased incidence of staphylococcic pneumonopathies in our environment,^{16, 17} its serious character, and the frequency of pleural complications, the prognosis of the disease is necessarily a reserved one.

The tetracycline-oleandomycin combination proved simple to administer to the child in syrup form. Tolerance for the medicament was excellent; neither vomiting, diarrhea, nor oral or intestinal moniliasis were observed, not even in the complicated cases in which treatment continued for more than one month.

TABLE II

Twenty-five Cases of

No.	Age	Weight, Gm.	% ideal weight	Days of disease	Condition		Roentgeno- grams		Oral tetracycline- oleandomycin				Penicillin	
					Serious	Mod. serious	Empy- ema	Perito- neum	Intra- venous initial	Daily dose, Gm.	Total dose, Gm.	Day	Total dose (million units)	Day
1	15 d.	2.200	70	6	+		Lt.			0.20	200	1	2	1
2	4 mo.	7.000	120	10	+			Rt.		0.5	7		56	14
3	5 mo.	5.200	85	21	+		Rt.			0.5	7	14	96	16
4	7 mo.	7.000	98	32	+			Rt.		0.5	2	4	50	34
5	12 mo.	6.500	70	20	+		Rt.			0.20	4.4	11	22	28
6	12 mo.	6.000	60	15		+	Rt.			1.2	22.5	15	69	20
7	18 mo.	10.300	102	10	+			Rt.		0.75	37	52	201	41
8	20 mo.	9.800	90	30	+			Rt.		1.00	30	30	30 Gm. erythromycin	30
9	24 mo.	10.000	90	11	+			Rt.		1.00	22	22	45	15
10	2.4 yr.	10.500	85	3	+			Lt.		1.00	47	47	90	30
11	3.9 yr.	15.000	100	16	+			Lt.		1.00	28	28	102	17
12	17 d.	3.500	100	4	+		Rt.		0.5x3	0.5	11.5	23		
13	1 mo.	2.100	65	5	+			Rt.		0.6-0.2	17.3	42		
14	2 mo.	3.700	87	8	+			Rt.	0.25x2	0.5	15	30		
15	3 mo.	6.900	130	6	+			Rt.	0.5x2	1.0	53	53		
16	5 mo.	5.900	75	4	+		Lt.		0.5x1	0.5	5.5	11		
17	8 mo.	6.300	98	4	+			Lt.	0.5x1	1.0	30	30		
18	16 mo.	8.400	80	22	+		Rt.		0.5x3	0.5	17	34		
19	1.8 yr.	5.800	55	2	+		Lt.		0.25x1	0.5	12	24		
20	1.8 yr.	10.000	92	4	+			Lt.	0.5x1	0.5	7	14		
21	1.9 yr.	10.000	91	17	+			Rt.		1-0.5	37	43		
22	2.9 yr.	12.300	92	3	+			Rt.	0.5x3	1.0	31	31		
23	2 yr.	10.600	95	3	+		Rt.		0.5x3	0.5	14.5	29		
24	2 yr.	10.400	94	32	+			Lt.	0.5x1	1.0	27	27		
25	3 yr.	12.000	90	9	+			Rt.	0.25x2	1.0	48	48		

Clinicoradiological progress of the patients with uncomplicated pneumonopathy was good. All of the children recovered, and the duration of the disease did not differ appreciably from that observed with other antibiotics.

Two cases in this group (cases 9 and 24), which ran a more prolonged course, were classified as being of staphylococcic etiology.

In the earlier patients with pleural empyema, the tetracycline-oleandomycin combination was supplemented by the administration of sodium penicillin (10 cases) or erythromycin (1 case) and/or intrapleural chloramphenicol (14 cases).

Numerous publications have suggested the use of combinations of antibiotics^{3, 5-7, 11, 16, 20} for staphylococcal empyemas, owing to the development of increased resistance of the bacteria to every new antibiotic used.

This year, we used the tetracycline-oleandomycin combination exclusively for

TABLE II

Pleural Empyema

Thoracentesis no.	Thorecotomy, days	Antibiotic intrapleural	Improvement, days	Fever, days	Radiological result	Final evaluation
1		Chloramphenicol 0.5		2		Autopsy
	14	Chloramphenicol 0.5x14	12	16	Opacity Pleur. min.	1 month
	9	Chloramphenicol 0.50x9	16	3	Completely clear	2 months
	8	Chloramphenicol 0.50x8		4		Autopsy
1		Penicillin 500,000 units	4	5	Opacity Pleur. min.	1 month
1		Chloramphenicol 0.25x1	3	3	Completely clear	2 months
5	12	Chloramphenicol 0.25x17	45	30	Completely clear	4 months
	30	Chloramphenicol 0.50x30		30	Fibrothorax (pneumonectomy)	6 months improved
	19	Chloramphenicol 0.50x19		32	Fibrothorax (decortication)	3 months improved
2	7	Chloramphenicol 0.50x9	15	29	Pneumothorax marginal	2 months
	14	Chloramphenicol 0.50x19	6	4	Completely clear	1 month
	4	Tetracycline-oleandomycin 0.1x4	10	2	Completely clear	1 month
	10	Tetracycline-oleandomycin 0.1x19	25	2	Completely clear	1.5 months
	19	Tetracycline-oleandomycin 0.1x19	6	1	Completely clear	1 month
	38	Tetracycline-oleandomycin 0.1x38	20	11	Completely clear	3 months
	3	Tetracycline-oleandomycin 0.1x3	10	6	Completely clear	1 month
	25	Tetracycline-oleandomycin 0.1x25	15	20	Opacity Pleur. min.	3 months
	8	Chloramphenicol 0.5x8	15	2	Completely clear	2 months
	9	Tetracycline-oleandomycin 0.1x9	4	3	Completely clear	1 month
	4	Tetracycline-oleandomycin 0.1x4	5	8	Opacity Pleur. min.	1 month
4		Chloramphenicol 0.5x4	10	20	Pneumatocele (resection)	1 month
	19	Tetracycline-oleandomycin 0.1x19	26	20	Pneumothorax Marginal	1 month
3		Chloramphenicol 0.5x2	10	10	Opacity Pleur. min.	1 month
	24	Tetracycline-oleandomycin 0.1x24	28	21	Fibrothorax Operative death	Autopsy
	23	Tetracycline-oleandomycin 0.1x21	20	30	Opacity Pleur. min.	2.5 months

the treatment of this serious disease, following a previously established plan that has been explained in detail before.

We regard the progress of these cases as satisfactory; the total mortality was 12 per cent.

The duration of the disease in the improved patients did not differ from that observed by us when using other antibiotics.^{2,15,17}

In our hospital environment, owing to the high rate of the incidence of staphylococcic empyema, at the present moment we must employ an efficacious antibiotic, the extended use of which will not result in any serious side reactions in the patients.

The oral tetracycline-oleandomycin combination is efficacious, it is easy to administer over an extended period of time, and it causes no side effects.

1. The authors analyze the results obtained by the use of the tetracycline-oleandomycin combination in 24 cases of uncomplicated acute pneumonopathy. Improvement was achieved in all these cases.

2. Twenty-five additional cases are described of staphylococcic empyema treated with the tetracycline-oleandomycin combination alone or in association with another antibiotic. Drainage of pleural exudate was effected by thoracentesis in 5 children and by thoracotomy with closed drainage in 20. Improvement without sequelae was achieved in 11 children, improvement with mild sequelae in 8, and surgery was performed in 3. Three children died.

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Two Hundred Cases of Bronchopneumonia Treated with a Combination of Oxytetracycline-Oleandomycin

J. G. PAGOLA, A. HEREDIA, AND J. CARRILLO

Department of Infectious Diseases, Children's Hospital, Mexico City, Mexico

One of the five principal causes of infant mortality in Mexico is pneumonia, which affects especially children under the age of 4.

Although bacterial pneumonia is the most frequent, numerous cases must be considered that could not possibly have been bacterial, such as pneumonia due to inhalation of foreign substances (oil, milk, petroleum, zinc stearate, etc.),¹⁻⁶ pneumonia originated by *Entamoeba histolytica*,⁷ and pneumonia due to interstitial plasma cells,^{8,9} which are prone to complication by secondary infections caused by the bacteria found in the respiratory tract. The use of antibiotics is therefore necessary in such cases. The clinical and radiographic diagnosis of pneumonia is quite simple but the precise bacteriological diagnosis in some cases is difficult to establish, since this group of diseases is caused by different organisms and various mixed infections.

After several bacteriological studies made at the Children's Hospital in Mexico City, it was found that the greater number of *Staphylococcus* isolates belong to type III. Analyzing sensitivity results with the commonly used agents, it was shown that of various strains of *Micrococcus pyogenes* var. *aureus* investigated, 80 per cent were resistant to penicillin, 60 per cent to tetracycline, 30 per cent to erythromycin, and only 5 per cent to oleandomycin.

Because of studies made on lung tissue at necropsies performed on patients who had died from bronchopneumonia (table I), we initiated a study of the value of a combination of oxytetracycline-oleandomycin* in the treatment of bronchopulmonary infections. This combination has shown a marked therapeutic value in several types of infectious diseases.¹⁰⁻¹²

MATERIAL AND METHODS

Two hundred children with severe infections of the lower respiratory tract were selected; these children had been admitted to the Children's Hospital during early 1959. Two groups of the same number were formed. The first group consisted of 100 patients with bacterial bronchopneumonia that had been contracted outside of the hospital and who had received no previous treatment. The ages of the patients ranged from 2 weeks to 4 years. Thirty per cent of the children were eutrophic, 46 per cent presented grade I hyponutrition and 24 per cent presented grade II hyponutrition. Diagnosis was made clinically, radiologically, and by laboratory tests. The second group consisted of 100 children ranging in age from 2 weeks to 4 years and who had contracted bronchopneumonia within the hospital and who had not responded to treatment with different antibiotics, especially penicillin, either alone or in combination with streptomycin or sulfonamides, over treatment

* The trade name of Chas. Pfizer & Co. for oxytetracycline-oleandomycin is Megamycin.

TABLE I

*Bacteriological Report on 48 Necropsies Effected at Children's Hospital
(Cause of Death: Bronchopneumonia)*

Microorganisms	Number of isolates
<i>M. pyogenes</i> var. <i>aureus</i>	17
<i>E. coli</i>	7
<i>Proteus</i>	5
<i>Streptococcus</i> , beta hemolytic	3
<i>K. pneumoniae</i>	3
<i>Streptococcus viridans</i>	2
<i>D. pneumoniae</i>	2
<i>Diplococcus diphtheriae</i>	1
<i>H. influenzae</i>	1
<i>Ps. aeruginosa</i>	1
<i>Neisseria flava</i>	1
<i>Histoplasma capsulatum</i>	1
<i>E. histolytica</i>	1
<i>Salmonella typhi</i>	1
<i>Salmonellae</i>	1
<i>Candida albicans</i>	1

periods of from three to seven days. Table II shows a classification of these cases according to the diagnosis and the total number of each type.

Fourteen per cent were eutrophic, 44 per cent presented grade I hyponutrition, 33 per cent presented grade II hyponutrition, and 9 per cent presented grade III denutrition.

All patients were treated with a combination of oxytetracycline-oleandomycin in a physical proportion of 2:1, available as an oral suspension; 50 mg./Kg. of body weight was administered over each 24 hour period, in four divided doses at intervals of six hours, for from five to seven days.

The etiological diagnosis was quite difficult to determine exactly with the exception of some cases in both groups in which the organism producing the disease was

TABLE II

Classification in Accordance with the Basis Used for Selection, Diagnosis, and Complications

Diagnosis	Number of cases
Disease contracted outside of hospital, not previously treated	
Bronchopneumonia	100
Disease contracted within hospital, treated previously	
Bronchopneumonia	49
Bronchopneumonia complicating whooping cough	17
Bronchopneumonia complicating laryngotracheobronchitis	10
Bronchopneumonia with severe infectious enteric diarrhea and electrolyte imbalance	6
Bronchopneumonia complicating congenital cardiopathy	5
Bronchopneumonia complicating bulbar poliomyelitis	3
Bronchopneumonia complicating leukemia	3
Bronchopneumonia complicating measles	2
Bronchopneumonia complicating Wilms's tumor	2
Bronchopneumonia and septicemia	1
Bronchopneumonia and meningitis	1
Bronchopneumonia and thrombophlebitis	1

isolated in cultures from exudates (nasal, tracheal, and bronchial) and blood cultures; a broad variety of organisms and frequently mixed flora were isolated in many cases. *M. pyogenes* var. *aureus* was the organism most frequently isolated; the next in importance was alpha hemolytic *Streptococcus*, followed by *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, beta-hemolytic *Streptococcus*, *Diplococcus pneumoniae*, *Neisseria meningitidis*, *Hemophilus influenzae*, *Escherichia coli*, and *Proteus*.

RESULTS

In the first group, the clinical symptoms disappeared or improved greatly in from two to three days in 55 per cent of the cases, and in an additional 34 per cent of the cases, in from the third to sixth days. In 8 per cent of the cases an indeterminate response was observed after the seventh day, and 2 patients died during the first 24 hours, before treatment could take effect.

In the second group, which had been treated previously with different antibiotics without satisfactory results, there was a complete abatement of all symptoms in 53 per cent of the cases after two or three days of therapy, and in an additional 30 per cent of the patients between the third and sixth days. The remaining patients received other methods of treatment. Eight patients died, a fact that can be explained by the presence of serious complications such as empyema and pulmonary abscesses. The combination of oxytetracycline-oleandomycin was tolerated satisfactorily and no toxic effects were observed that could be attributed to the administration of the drug.

DISCUSSION

In the treatment of bronchopneumonia, all the available antibiotics are, of course, used by us, but the pediatrician is faced with a very special problem due to the fact that with these infections it is difficult to establish a precise etiological diagnosis, since such a large variety of pathogenic microorganisms is involved, with a predominance of *M. pyogenes* var. *aureus*, beta-hemolytic *Streptococcus*, *D. pneumoniae*, and *H. influenzae*. We believe that the treatment of bronchopneumonia should be specific when the isolation of the organism and the determination of susceptibility are possible, but one must take into consideration the fact that the etiological diagnosis of infections of the lower respiratory tract presents problems that often cannot be solved in private practice.

The results of cultures obtained in postmortem examinations of pulmonary tissue of children with poliomyelitis who had died from bronchopneumonia have been related to organisms isolated from the upper respiratory tract.¹³ In our experience the cultures obtained have shown a great variety of pathogenic organisms, which enables us to infer that the use of oxytetracycline-oleandomycin is fully justified in the treatment of cases of bacterial pneumonia associated with bronchitis, since its components cover the infecting flora selectively.^{11, 14, 15}

The response to treatment with oxytetracycline-oleandomycin in the patients with bronchopneumonia included in this study was different in each group, which was to be expected in view of the basis used for the selection of cases. The chil-

dren included in the first group were treated in due time and had contracted the disease outside of the hospital. The second group was composed of children who had contracted the disease inside the hospital, but their treatment with the combination was begun late, after other antibiotics were first administered and they had failed to respond to these agents. In addition, the prognosis in these cases was unfavorable due to the poor state of nutrition and to the fact that, in the majority of the cases, there were concomitant diseases.

The mortality can be explained by the fact that in cases of staphylococcal pneumonia, if antibiotic treatment is not started soon enough, complications such as empyema and lung abscesses may develop.

These facts emphasize the importance of early treatment of bacterial bronchopneumonia with antibiotics effective against all the different infecting organisms. This procedure avoids a successive change of antibiotics and the possibility of superinfection.

The problem of bacterial resistance with reference to *Staphylococcus* has received special attention in hospital practice, where it occurs most frequently. It has been established that each hospital is characterized by the development of its own special strains, which are carried to the patients by the hospital personnel, and this also partially explains the differences in the results obtained in this study and emphasizes the advantages of the use of oxytetracycline-oleandomycin in private practice.

SUMMARY

Two hundred children with bronchopneumonia of varied microbial etiology including some with severe concomitant diseases were treated orally with a combination of oxytetracycline-oleandomycin at a dosage of 50 mg./Kg. of body weight daily. Of a series of 100 patients who had contracted the infection outside of the hospital, satisfactory results were obtained in 90 per cent of the cases. Of a second series of 100 children who had contracted the disease within the hospital, the results were satisfactory in 83 per cent of the cases.

CONCLUSIONS

1. Due to the fact that pneumonia belongs to a group of diseases caused by different pathogenic organisms, the prognosis of which depends on the rapidity with which proper antibiotic treatment is established, the practitioner is frequently obliged to act before determining a precise etiological diagnosis.

2. Adequate treatment depends on the administration of an antibiotic preparation effective against the wide variety of infecting organisms. For this reason we feel that the oxytetracycline-oleandomycin combination is the drug of choice in the therapy of bacterial bronchopneumonia.

3. In cases in which the determination of the etiological agent and its sensitivity is possible, antibiotic treatment should be specific.

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Effect of Triacetyloleandomycin on Bacterial Infections of the Respiratory Tract

H. WAREMBOURG

Professor of Therapeutics at the Lille Medical School, Physician of the Calmette Hospital, Lille, France

This study was undertaken to evaluate triacetyloleandomycin* in the treatment of some bacterial infections of the respiratory tract. The worthwhile effect of this antibiotic on pulmonary diseases has been already described. It was investigated in pneumonia by Isenberg and Karelitz¹ and by Kaplan and Goldin² in bronchial asthma.

TECHNIQUE

We have studied 38 adult patients with various infections of the respiratory tract. They were classified as follows: (1) Episodes of acute or subacute bronchitis associated with chronic bronchial suppuration (with or without bronchiectasis) or chronic emphysema; (2) pulmonary abscess; (3) acute diseases of the lung, such as pneumonia; (4) diffuse bronchoalveolitis secondary to influenza; (5) superinfection of pulmonary tuberculosis by acute episodes of common bronchitis; and (6) infection superimposed upon bronchogenic carcinoma.

Patients were checked by clinical, roentgenograms, and laboratory examinations (studies of the bacteria in the sputum and of sensitivity to antibiotics, of the sedimentation rate, of the blood counts, and of the plasma proteins.)

Bronchoscopies and lipiodol studies were performed as often as necessary. Renal and hepatic tests were performed (urinalysis, chemistry, and cytology; blood urea; Gros' test; Hanger's test; Mac Lagan's test; cholesterol ratio; and induced galactosuria).

Triacetyloleandomycin was given orally at a daily dosage of 2 Gm. divided into four doses of 0.50 Gm. each, taken between meals. In some instances, because of some digestive disturbances, 1 Gm. a day was given. The length of the treatment period depended upon the type of the disease and on the results obtained; it ranged from 10 to 60 days. Therefore, the total dose administered ranged from 20 to 140 Gm.

Usually, triacetyloleandomycin was the only antibiotic given. In 6 cases of severe infections, we used a combination of other antibiotics, mainly penicillin and streptomycin.

RESULTS

The results will be described in each of the six groups of our classification.

Episodes of Acute or Subacute Bronchitis Associated with Chronic Bronchial Suppuration (with or without Bronchiectasis) or Chronic Emphysema. There were

* The trade name of J. B. Roerig Division, Chas. Pfizer & Co., for triacetyloleandomycin is Tao.

11 patients in the group. Five patients were febrile; fever disappeared after administration of triacetyloleandomycin. Seven of 10 patients noticed the disappearance or an important diminution in their expectorations. The erythrocytes, sedimentation rate, the leukocytosis, and the albumin/globulin ratio of the blood proteins became normal in 4 of 8 patients. Improvement was good for 10 of the 11 patients.

Superinfection of Chronic Pulmonary Tuberculosis by Acute Episodes of Common Bronchitis. There were 10 patients in this group. Fever disappeared after administration of triacetyloleandomycin in 4 of 6 patients, the expectoration decreased in 8 of 10. The sedimentation rate, blood count, albumin/globulin ratio became normal in 5 of 8 patients. The control of the superinfection was good in 9 of 10 cases.

Acute Diseases of the Lungs, such as Pneumonia. There were 4 patients in this group. Temperatures went down to normal in 24 to 48 hours. Expectoration disappeared in the only case where it was noticeable. For the 4 patients, the sedimentation rate became normal as well as the roentgenograms. All the patients experienced complete cure.

Pulmonary Abscesses. There were 4 patients in this group; 3 were infected with β -hemolytic *Streptococcus* and pneumococcus, and 1 with Friedlander's bacillus. Fever disappeared in 3 of 3 patients, expectoration disappeared in 4 of 4, and roentgenograms improved for 3 of 4. Sedimentation rate returned to normal in 3 of 4. For all cases, improvement was good but in each case triacetyloleandomycin was combined with penicillin and streptomycin.

Diffuse Bronchoalveolitis Secondary to Influenza. There were 4 patients in this group. Fever subsided in all cases, with return of the sedimentation rate to normal. Improvement was very good.

Infection Superimposed upon Bronchogenic Carcinoma. There were 4 patients in this group. Disappearance of the fever was observed in 2 of 3 patients, the expectoration disappeared for 2 of 3, the sedimentation rate returned to normal in 2 of 4. For one of the patients the roentgenogram showed a slight improvement. For 3 of 4 patients, the infectious complication was markedly improved.

DISCUSSION

The following conclusions may be drawn from our data.

For all patients with respiratory infections, the drug produced marked improvement in the clinical course. The improvement of the fever was particularly good in 21 of 24 patients, with a rapid drop to normal (24 to 48 hours). Decrease in the amount of expectoration took place with disappearance in 9 of 20 and a marked diminution in 11 of 20. More than half of the patients showed general improvement with a gain in weight from 1 to 8 Kg.

Results were especially striking roentgenographically in the pneumonia cases with a complete and rapid clearing of the pulmonary opacities. For 2 cases, bronchoscopy and bronchography showed that all the bronchi were normal at the end of the treatment.

The sedimentation rate returned to normal in all cases in which it had previously been elevated except for 3 patients. The polymorphonuclear leukocytosis was less improved, with a decrease in only 5 of 10 patients. The initial abnormalities of the

blood proteins (decrease of albumin, increase of alpha 1, alpha 2, and sometimes gamma globulins) disappeared in 5 of 7 patients.

Before administration of the drug, the following bacteria were found in the sputums: *Diplococcus pneumococci* (12), streptococci (25), staphylococci (4), *Neisseria catarrhalis* (24), *Escherichia coli* (4), *Hemophilus influenzae* (4), *Proteus hauseri* (3), Friedländer's bacillus (1), *Alcaligenes* (1). The strains of pneumococci, streptococci, staphylococci, *N. catarrhalis* and *H. influenzae* were sensitive to triacetyloleandomycin in vitro. But *Klebsiella pneumoniae* and *Escherichia freundii* were not influenced. Two of 3 strains of *Alcaligenes* were not sensitive. Following treatment with triacetyloleandomycin, there was a complex modification of the bacterial spectra in the sputums. Pneumococci disappeared in 5, streptococci in 9, staphylococci in 1, *H. influenzae* in 2, *Klebsiella aerogenes* in 1 and *N. catarrhalis* in most of the patients. On the other hand, for some of the patients, treatment with triacetyloleandomycin was followed by appearance in the sputum of new bacterial species: *Proteus hauseri* in 2, *E. coli* in 1, *H. influenzae* in 1 and *K. aerogenes* in 1.

From the data outlined, it can be said that the treatment of our patients with infection of the respiratory tract with triacetyloleandomycin: (1) Was completely successful in 29 patients (76.3 per cent) when used alone. (2) Was successful in 6 patients when combined with other antibiotics. The evaluation of its effect in these patients is therefore difficult; nevertheless if we add these patients to those treated with triacetyloleandomycin alone, our clinical success rate is 92 per cent. (3) Failed 3 times (8 per cent).

The tolerability of the drug was good. Some disturbances of the digestive tract were observed, nausea fairly frequently, rarely vomiting (in 1 case). These side effects appeared either in the initial treatment period (third or fourth day) or in the latter part of the treatment period; tenth day in 2 patients and twentieth day for 3 patients. Often a slight decrease in the dosage of triacetyloleandomycin caused the side effects to disappear. In only 4 patients was it necessary to discontinue treatment.

There was no sign of impairment of the hepatic, renal, or blood functions.

CONCLUSION

From our study it is apparent that triacetyloleandomycin is a very effective and well tolerated antibiotic for patients with bacterial infections of the respiratory tract. In mild infections, it is successful when used alone. In severe infections, it is frequently combined with other antibiotics, i.e., penicillin and streptomycin. This drug is of particular value in the treatment of patients infected with organisms resistant to other commonly used antibiotics.

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Effectiveness of Oral Erythromycin Propionate in Commonly Occurring Infections

BENJAMIN CALESNICK

Laboratory of Human Pharmacology, Hahnemann Medical College, Philadelphia, Pa.

Erythromycin has been used successfully by the oral route for the same bacterial spectrum as penicillin.¹ Yet, various chemical complexes have been prepared in an attempt to improve its clinical effectiveness by increasing the blood level. The most promising derivatives have been various esters, since the salts formed by mineral acids are chemically unstable. Erythromycin propionate* has been shown to produce higher blood levels than either the base¹ or erythromycin stearate.² This study was initiated to determine, first, the responsiveness of acute infections, which are commonly encountered, to the oral administration of erythromycin propionate, and, second, any clinical advantage that this preparation has over the base.

A group of 127 patients with acute infections were followed during the period between January 1 and August 15, 1959. The ages ranged from 6 to 67 years and were equally represented by both sexes. They were seen in office medical and dental practices, outpatient departments, and as in-patients of various institutions. Approximately 98 per cent were ambulatory.

These patients were divided into two therapeutic groups. Group 1 comprised the infections that were not readily amenable to bacteriological studies, i.e., sinusitis, otitis media, cellulitis, and adenitis. Group 2 included those diseases in which material for bacteriological studies was easily available, i.e., pharyngitis, tonsillitis, exudative pulmonary states, furunculosis.

All patients were examined daily. Members of group 1 received their medication on the first visit, whereas the members of group 2 had smears taken on their first visit and then were placed on a placebo until the bacteriological studies were completed. Following this, only the individuals with erythromycin-sensitive organisms were given erythromycin propionate. This drug was administered in capsules and both groups were instructed to take medication every six hours. Individuals weighing less than 100 pounds received a daily dose of 500 mg.; those weighing more than 100 pounds received a daily dose of 1 Gm.

The duration of therapy depended on the diagnosis. Each patient, without a bacteriological survey, was administered the drug for a six day period. No time limit was set for the other group. Since in this study erythromycin propionate was used for short-term therapy, no toxicity studies were performed.

All the bacteriological studies were performed in the Laboratory of Human Pharmacology and the Microbiology Laboratory at the Hahnemann Medical College. The program was set up first to isolate the offending organisms and then to determine their sensitivity to erythromycin. Standard bacteriological methods were used, including 2 μ g. erythromycin discs for sensitivity testing.

* The trade name of Eli Lilly & Co. for erythromycin propionate is Ilosone.

TABLE I

*Results Obtained in Various Acute Infectious States
without Bacteriological Studies*

Diagnosis	Number of patients	Clinical response		
		None	Partial	Complete
Sinusitis	17	0	4	13
Otitis media	11	0	3	8
Adenitis	7	0	4	3
Cellulitis	6	0	1	5
Enteritis	2	2	0	0
Total	43	2	12	29

RESULTS

Therapeutic. GROUP 1. The responses of the 43 patients are tabulated in table I. As can be seen, 67.4 per cent of these patients were completely cured in the six day period. It seems probable that if therapy was continued for a longer time many of the remaining 33.6 per cent would have also shown complete remission.

GROUP 2. The results of the 84 patients who received a complete bacteriological evaluation are tabulated in table II. A further breakdown of these cases appears in table III, where the offending organisms are tabulated. As can be seen, alpha- and beta-hemolytic *Streptococcus* and *Staphylococcus aureus* accounted for 71 per cent of the organisms isolated in commonly occurring infections. The in vitro sensitivity to erythromycin of these three microorganisms varied between 76.5 and 96.3 per cent. On the other hand, 81.9 per cent of all the organisms isolated were erythromycin sensitive. It is of interest to observe that this drug was most effective against alpha-hemolytic *Streptococcus*, which was the most common organism isolated. Although erythromycin propionate was administered only to patients who had erythromycin-sensitive organisms, 6 therapeutic failures were observed. However, further

TABLE II

Disease States in Which a Complete Bacteriological Evaluation Was Obtained

Clinical diagnosis	Number of cases	Organisms isolated
Pharyngitis	31	<i>N. catarrhalis</i> , Yeast, alpha- and beta-hemolytic streptococci
Tonsillitis	27	<i>Staph. aureus</i> , <i>Staph. albus</i> , and <i>Staph. citreus</i>
Furunculosis	15	<i>Staph. aureus</i> , <i>Staph. albus</i> and <i>Staph. citreus</i> , <i>Staphylococcus</i> (coagulase negative), <i>Pseudomonas</i>
Bronchitis	11	<i>H. influenza</i> , alpha- and beta-hemolytic streptococci
Apical abscess	5	<i>Staph. albus</i> and <i>Staph. aureus</i>
Bronchiectasis	3	<i>Klebsiella</i> , alpha- and beta-hemolytic streptococci
Lobar pneumonia	1	<i>Pneumococcus</i>
Tracheobronchitis	1	Beta-hemolytic streptococci
Pyelonephritis	1	<i>E. coli</i>
Total	84	

TABLE III

Bacteriological Study of Commonly Occurring Infections

Organisms	Cultures		In vitro sensitivity to erythromycin %
	Number	%	
<i>Streptococcus</i>			
alpha-Hemolytic	33	27.2	96.3
beta-Hemolytic	31	26.6	76.5
<i>Staphylococcus aureus</i>	22	18.2	88.9
<i>Neisseria catarrhalis</i>	12	9.9	41.7
<i>Staphylococcus albus</i>	7	5.8	85.7
<i>Staphylococcus citreus</i>	4	3.3	75.0
<i>Staphylococcus</i> (coagulase negative)	4	3.3	100.0
Yeast	3	2.5	0.0
<i>E. coli</i>	1	<1.0	0.0
<i>H. influenza</i>	1	<1.0	100.0
<i>Klebsiella pneumoniae</i>	1	<1.0	100.0
Pneumococcus	1	<1.0	100.0
<i>Proteus vulgaris</i>	1	<1.0	0.0
<i>Pseudomonas aeruginosa</i>	1	<1.0	0.0
Total	122		

studies revealed that these patients were "carriers" since 3 developed virus pharyngitis, 2 developed infectious mononucleosis and 1 developed measles. The average time for recovery was two to three days.

Side Effects. Side effects developed in 6 of the 127 cases in this study. Aphthous ulcers developed in 1 patient (9 year old boy) and the other side effects occurred only in adult women. Two complained of nausea and 3 complained of loose bowels. Thus, the incidence of these untoward reactions was 6/127 or 4.7 per cent.

DISCUSSION

In the present study of commonly occurring infectious states, it was found that the clinical effectiveness of erythromycin propionate was, of course, much greater when erythromycin-sensitive organisms were involved. In the group of patients who had a bacteriological evaluation, complete recovery was obtained in 92.8 per cent within three days. This high degree of effectiveness might be due to the rapid and prolonged therapeutic blood levels that Griffith obtained with this agent.¹

Side effects, of the gastrointestinal variety, produced by the use of erythromycin base have been reported to occur in 7.8 per cent by Cronk and Naumann,³ 31.5 per cent by Batterman et al,⁴ and 39 per cent by Shlaes et al.⁵ In the present study, using erythromycin propionate, the over-all incidence of side effects was only 4.7 per cent, which compares favorably with the findings of others. Perry et al⁶ observed no side effects in their group, Smith and Soderstrom⁷ reported 7.6 per cent, and Griffith¹ reported an incidence of 8.6 per cent. Thus, it appears that esterification of erythromycin with propionic acid results in a compound with an appreciably lower toxicity than the parent compound.

SUMMARY

1. Erythromycin propionate was administered by mouth to 127 patients with commonly occurring infections. When patients with erythromycin-sensitive organisms were used, 92.8 per cent had complete recovery in two to three days. If there was no bacteriological evaluation of the infections, 67.4 per cent of the patients had a complete cure in the six day period and 28.0 per cent had a partial cure.

2. In contrast to erythromycin base, this ester has a lower incidence of side effects when used for short-term therapy.

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The Use of a Tetracycline-Oleandomycin Preparation in the Treatment of Cholecystitis and Cholangitis

M. STEFAN GREIF

Medical Director, Second Medical School, Landeskrankenhaus, Graz, Austria

Because of the possibility of serious complications in inflammatory disease of the biliary passages (cholecystitis and cholangitis), effective antibiotic therapy is frequently urgently indicated. I should like to report here on the results in cholecystitis and cholangitis of the intravenous administration of a tetracycline-oleandomycin preparation.* A survey of the literature reveals no large scale investigations of the administration of this antibiotic preparation in inflammatory biliary tract disease.

A number of investigators have studied the concentration of tetracycline in the duodenal fluid after intravenous administration. The results of such investigations indicate that in gallbladder inflammation in the absence of obstruction of the biliary tract, tetracycline administered by the intravenous or intramuscular route can be detected in the bile in concentrations greatly exceeding those necessary for therapeutic effect. In cases in which the cystic duct was obstructed, tetracycline concentration in the bile following intramuscular or intravenous injection was significantly lower than in the unobstructed biliary system, yet these concentrations were high enough to be therapeutically effective. Likewise, we were aware of a number of favorable reports of the synergistic effect of tetracycline and oleandomycin against various microorganisms in vitro. On the basis of these laboratory findings, concerned on the one hand with the effect of tetracycline alone and on the other with the synergistic effect of tetracycline-oleandomycin, we undertook a large scale trial of the effect of tetracycline-oleandomycin in inflammatory diseases of the biliary tract.

METHOD OF INVESTIGATION

To attain an objective evaluation of the effect of tetracycline-oleandomycin in cholecystitis and cholangitis the following plan of investigation was followed.

In patients suffering from biliary tract inflammations, bile was obtained by duodenal drainage on three successive days and examined bacteriologically. For the present investigation, only those cases were selected that showed the same cultural findings on three consecutive days. This restriction was made because of our experience that a single positive bacteriological finding on bile, obtained by duodenal drainage, does not constitute proof of the bacterial etiology of the biliary inflammation. I do not wish at this time to go into detail about this point but refer you to the relevant literature. Only repeated consistent demonstration of specific bacteria in biliary fluid can be accepted as evidence of infection of the biliary passages. We feel that the method we have employed establishes, prior to the initiation

* The trade name of Chas. Pfizer & Co. for a 2:1 combination of tetracycline and oleandomycin is Signemycin.

of therapy, the presence of an inflammation of infectious etiology. The bacteria thus recovered were immediately tested according to conventional laboratory procedures to ascertain their sensitivity to tetracycline-oleandomycin.

Roentgenographic examination of the gallbladder and biliary passages performed at the same time demonstrated that in some of the patients the gallbladder and biliary tree could be roentgenographically demonstrated; in others the biliary system could not be visualized. These roentgenographic findings, however, did not influence our decision for or against administration of tetracycline-oleandomycin, because, as is generally recognized, neither positive nor negative findings give reliable information about the degree of inflammation or the infectious etiology of such inflammation.

Our investigations were performed in such a way that those patients in whom bile examination on three consecutive days revealed bacteria sensitive to tetracycline-oleandomycin then received tetracycline-oleandomycin therapy. These patients were given 250 mg. of tetracycline-oleandomycin by the intravenous route twice daily for four or five days. After that period of therapy, bile was again obtained by duodenal drainage and examined bacteriologically. When bacteriological findings were negative, therapy was terminated and a second drainage and bacteriological examination was performed three or four days after cessation of therapy. Patients, in whom both examinations were negative, did not receive any further medication. In the patients in whom bacteriological findings were positive on the first examination, therapy was continued for an additional three or four days. At this time the second bile culture was performed. If, after this second period of therapy, the bile had become bacteriologically negative, therapy was discontinued and a final follow-up examination was performed after a lapse of three or four days without therapy. When this examination proved negative, the patient was discharged. If, however, after such protracted therapy, bacteriological findings would continue to be positive, we were prepared to stop therapy and consider the treatment regimen to be demonstrated as being ineffective.

RESULTS

In 80 patients pretreatment examinations revealed the presence of staphylococci and streptococci in the bile on three consecutive days. These bacteria all proved sensitive to tetracycline-oleandomycin *in vitro*. Consequently, tetracycline-oleandomycin was administered intravenously in a dosage of 250 mg. twice daily for four or five days. In all instances throughout the study the antibiotic was given in combination with a cholagogue.

Following this period of therapy, cultures of the bile were negative in 78 patients; these negative culture findings persisted on repeat culture three days later. In 2 patients the first post-treatment bile culture showed streptococci and staphylococci to be present. Therefore, therapy was continued for an additional four days. After this additional therapy, bile cultures became negative on the second post-treatment bacteriological test, a finding which was confirmed on the final follow-up examination three days later.

In summary, all 80 patients harboring streptococci and staphylococci in the bile were bacteriologically sterilized by tetracycline-oleandomycin administered intra-

venously. Six months later follow-up studies of 36 patients revealed persistence of negative bile cultures.

In 40 additional patients bacteriological examination of the bile revealed colon bacillus as well as staphylococci and streptococci. Antibiotic sensitivity testing of the bacteria in these cases showed all the staphylococci and streptococci to be sensitive to tetracycline-oleandomycin. In 30 patients the *E. coli* were less sensitive to tetracycline-oleandomycin than the cocci; and in 10 patients *E. coli* were found to be resistant in vitro. Following biliary drainage and culture of the bile, therapy was initiated in the same manner as with the 80 previously discussed patients. Following administration of 250 mg. of tetracycline-oleandomycin intravenously twice daily for five days, neither staphylococci nor streptococci were detectable on repeat culture. Moreover, on this immediate follow-up examination, no *E. coli* were recovered from 36 patients. These findings persisted on repeat culture five days later. In these patients a further reexamination after 14 days also revealed completely negative findings. In 4 cases the initial post-treatment examination five days after the initiation of therapy showed an elimination of staphylococci and streptococci from the bile; *E. coli*, however, persisted. In these cases therapy was continued for an additional five days during which 250 mg. tetracycline-oleandomycin was given twice daily as an infusion (60 drops per minute); post-treatment bile cultures in these cases revealed the continuing presence of colon bacilli and therapy was discontinued as ineffective.

In 21 of the 40 patients of this group bacteriological follow-ups were performed six months after therapy. In 19 of the patients so examined bile culture was sterile. In only 2 patients were *E. coli* once again detectable in the bile. These, however, disappeared after a repeated course of tetracycline-oleandomycin.

SUMMARY

Investigation of 80 patients demonstrated that where streptococci and staphylococci constituted the only bacterial flora of the bile, four or five days of tetracycline-oleandomycin given in a dosage of 250 mg. twice daily, intravenously, produced sterilization of the bile. When, in addition, the colon bacillus was also present, the bile was sterilized of streptococci and staphylococci by the same treatment and in 36 of these 40 patients their bile cultures were also sterilized of the colon bacillus. Follow-up examinations six months later revealed no instances of the reappearance of streptococci or staphylococci. In 2 cases, *E. coli* reappeared but disappeared upon renewed tetracycline-oleandomycin therapy. On the basis of these results, we are of the opinion that when staphylococci, streptococci, and/or colon bacilli are demonstrated in the bile, intravenous therapy with tetracycline-oleandomycin should be instituted.

Long-Term Treatment of Chronic Urinary Tract Infections

S. RUMMELHARDT

Urologic Ward of the First Surgical Clinic, University of Vienna, Vienna, Austria

Within the last two years, 65 patients suffering from chronic urinary tract infections had undergone a long-term therapy (table I) with several antimicrobial agents.

As with every group of urologic patients, men are predominant. The cases involved were mainly secondary infections of the urinary tract, which especially occurred in postoperative cases, e.g., following renal operations due to stones, or following surgery of the bladder due to papillomas or hypertrophy of the prostate. The secondary infections in patients not requiring surgical treatment were caused by prostatitis or urethritis. One woman who exhibited a primary infection of the urinary tract also had tuberculosis of the urinary tract.

The combination chosen when we started with a long-term treatment two years ago was oxytetracycline and a sulfonamide with a lasting effect (= 3-sulfanilamido-6-methoxypyridazine). Later on the patients had to be changed to cycloserine, another sulfonamide with a lasting effect (= 2,4-dimethoxy-6-sulfanilamido-1,3-diazine), and nitrofurantoin, partly because the sulfonamide given at first was no longer available and partly because the chosen therapy did not bring about any effect. Usually we initiate the long-term treatment with oxytetracycline and a sulfonamide with a lasting effect and then continue the therapy with one or several of the indicated preparations (table II).

As usual, infections of the urine due to coli bacilli were dominating. Of the drugs used, cycloserine often was not well tolerated (table II). In one case the sulfonamide had to be changed for another that was tolerated well. In another case oxytetracycline had to be withdrawn because of diarrhea. Failures were observed in infections caused by coli bacilli and *Proteus* organisms, whereas the pure cocci infections responded well. Cycloserine apparently produces a favorable effect in *Proteus* infections.

To judge the effect of the drugs used, the urine was bacteriologically tested and the sediment examined monthly. When judging the results from the clinical viewpoint, greater importance is attached to a urine free from inflammatory signs than to an eventual bacteriuria still existing alone.

In 16 patients (24.6 per cent) a sterile and cell-free urinary sediment was achieved. In 10 patients (15.4 per cent) the bacteriological examination revealed a cell-free sediment, yet the urine was not sterile. In those 27 patients (41.5 per cent) who showed subjective improvement because there was no change of the severe inflammation already present in the pyelogram, the residual nitrogen returned to normal, and the general physical condition improved so that the patient became capable of work though there were abundant germs in the urine, the state of infection was rated as "improved." Twelve patients (18.5 per cent) failed to respond to the long-term treatment.

Haschek and Ehrenreich¹ reported on 21 patients who were treated with a tetracycline and a sulfonamide with a lasting effect; 12 out of these 21 patients were refractory to therapy. Leff² used a sulfonamide with a lasting effect in 35 paraplegics

TABLE I
Long-Term Treatment in 65 Urological Patients

Sex	No.	Infection	
		Primary	Secondary
Men	55	4	51 { postoperative cases 37 no surgical intervention 14
Women	10	1	9 { postoperative cases 8 no surgical intervention 1

and during six months of therapy did not observe any further pyelonephritic exacerbations. Finally, Hohenfellner³ described 32 patients who were treated with oxytetracycline and a sulfonamide; 7 of these did not show any response.

From more extensive experiences with the long-term treatment of chronic urinary tract infections that were mainly caused by pyelonephritic changes of the kidneys, it was obvious that the decision whether complete recovery was already achieved is as difficult as in the case of tuberculous diseases of the kidney. Since, as is known, the mechanisms of origin of the specific and nonspecific chronic urinary tract infection are being compared with each other, a comparison of the method of treatment of either disease is justified.

According to the results so far obtained in chronic urinary tract infections, especially in chronic pyelonephritis, an alternating treatment with the afore-mentioned drugs is recommendable and on finally judging the results obtained with the long-term treatment in the unspecific infection the same standard is to be applied as to tuberculosis.

TABLE II
Drugs Used for the Long-Term Treatment of Infections Caused by Various Microorganisms

Drugs used	Good response	No response	Side effects	Germs					
				Coli bacilli	<i>Proteus vulgaris</i>	<i>Staph. albus</i>	<i>Staph. aureus</i>	<i>Streptococcus</i>	Mixed infections
Oxytetracycline: 35 times 2 × 100 mg. daily	27	8	(1)	7	—	4	—	1	4
Cycloserine: 29 times 2 × 250 mg. daily	22	7	(3)	5	1	3	1	—	3
Sulfonamide A*: 25 times 1 × 0.5 daily	22	3	(—)	3	1	2	—	—	3
Sulfonamide B†: 16 times 2 × 0.5 daily	13	3	(1)	3	—	1	—	—	1
Nitrofurantoin: 11 times 1 × 100 mg. daily	8	3	(—)	2	2	—	1	—	2
Bacteriological findings				43	18	24	10	3	27
No response				20	14	10	2	1	13

* Sulfonamide A = 2,4-dimethoxy-6-sulfanilamido-1,3-diazine.

† Sulfonamide B = 3-sulfanilamido-6-methoxypyridazine.

SUMMARY

This study reports 65 patients with chronic urinary tract infections who underwent long-term treatment. For two years therapy with oxytetracycline, cycloserine, sulfonamides, and nitrofurantoin, alone or in combination, has been given. Clinical recovery (sterile or cell-free urine) was achieved in 26 patients (40 per cent), whereas 27 patients (41.5 per cent) exhibited an improvement of the general physical condition and at least did not show any objective deterioration. Twelve patients (18.5 per cent) failed to respond.

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The Tetracyclines in Amebiasis

R. ELSDON-DEW, A. J. WILMOT, AND S. J. POWELL

Department of Medicine, University of Natal, and Amoebiasis Research Unit,
Durban, Union of South Africa*

The place of the tetracyclines in the treatment of acute amebic colitis is well established,¹⁻⁵ and though their action is probably indirect, they are among the most potent drugs available. This paper reports further experiences in the use of these antibiotics in various forms and dosages and in combination with other agents.

The assessment of the value of any therapy in amebiasis is set about with difficulties. The primary difficulty is lack of appreciation of the relationship between parasite and host. It has now been established⁶ that *Entamoeba histolytica* can exist in commensal form and that there must be some "trigger" that converts this commensal, possibly normal, form into the invasive form, which gave the parasite its name. A further difficulty lies in the recognition of the parasite. It has now been established⁷ that *Entamoeba hartmanni* may often have been mistaken for *E. histolytica*, casting doubt on the significance not only of many diagnoses and surveys, but also of many trials of therapy. It is not surprising therefore that there are such large differences in the assessment of drugs by different observers.

MATERIALS AND METHODS

The present series of trials is based on the treatment of acute amebic dysentery in Durban Africans,⁸ a condition in which there is no doubt of the connection between the parasite and the disease. There are still some 2500 cases seen at our hospital each year, providing an unparalleled opportunity for investigation. This plethora of cases has allowed stringent criteria in the selection of patients for trial therapy, but with the provision that when a drug fails to evoke early clinical improvement, the danger to life is such as to make imperative a change to the best-known form of therapy.

Briefly, our criteria, which have been set out in several previous papers,^{1,2} are: diarrhea with blood and mucus, ulceration visible at sigmoidoscopy, the presence of hematophagous amebas, and the absence of complications or intercurrent disease. Patients are hospitalized for 28 days; serial sigmoidoscopy at five day intervals and the daily examination of stools or scrapings permit us to follow the progress of the condition. Follow-up is based on monthly re-examinations when possible. This system is not altogether satisfactory, for the African is not only reluctant to lose a day's pay in the process, but in the meantime has probably returned to his previous conditions of bad hygiene and malnutrition, so that recurrence is likely.

* The Amoebiasis Research Unit is sponsored by the following groups: The South African Council for Scientific and Industrial Research, The Natal Provincial Administration, The University of Natal, and The United States Public Health Service (grant E-1592).

TABLE I
Therapeutic Trials

Series no.	Drug	Daily dosage	Days of therapy	No. of cases	Results at 28 days, %		
					Success	Doubtful	Failure
17	Chlortetracycline	1 Gm.	15	51	94	2	4
22	Oxytetracycline	1 Gm.	15	51	92	—	8
55	Chlortetracycline	80 mg.	10	19	47	6	47
53	Oxytetracycline residue	4 Gm.	10	10	60	10	30
57	Chlortetracycline residue	4 Gm.	10	14	79	—	21
59	Tetracycline	1 Gm.	15	36	97	—	3
58	Oxytetracycline, intramuscular	200 mg.	10	5	40	20	40
74	Diiodohydroxyquinoline Chloroquine sulfate	1800 mg.	20	25	20	16	64
		1200 mg.	1				
		400 mg.	14				
75	Tetracycline Diiodohydroxyquinoline	150 mg.	10	25	60	12	28
		1800 mg.	20				
77	Tetracycline Chloroquine sulfate	150 mg.	10	25	48	4	48
		1200 mg.	1				
		400 mg.	14				
79	Tetracycline Diiodohydroxyquinoline Chloroquine sulfate	150 mg.	10	50	94	—	6
		1800 mg.	20				
		1200 mg.	1				
		400 mg.	14				
87	Demethylchlortetracycline	600 mg.	10	12	33	8	58

RESULTS AND COMMENT

Table I shows the results of some of our therapeutic trials. When early trials indicate that a drug is unlikely to fall into the competitive range for its class, the number of patients treated is curtailed below the 50 we originally tested with each drug. As a baseline, periodic retrials of emetine are carried out to ensure that there has been no gradual change in the over-all picture of the disease. In our hands this drug has an approximate 27 day success rate of 50 per cent.

Chlortetracycline,^{1*} oxytetracycline,^{3†} and tetracycline^{4,5‡} each had a success unprecedented for any single drug, but they failed to prevent visceral complications;⁹ there was a relapse rate of the order of 15 to 20 per cent over three months, and also these drugs are expensive. Regarding the latter, we tested the residues from the manufacture of these products, at the same time as a lower dosage schedule. While these regimens gave a good end result, the rate of healing as shown by sigmoidoscopy was much slower, and the frequency of complications and relapses was of the same order. In addition, there was an appreciable conversion to cyst-passing, a phenomenon we had noted with other antibiotics, such as chloramphenicol.

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin.

† The trade name of Chas. Pfizer & Co. for oxytetracycline is Terramycin.

‡ The trade name of Lederle Laboratories Division, American Cyanamid Co., for tetracycline is Achromycin; of Chas. Pfizer & Co., Tetracyn.

TABLE II

Follow-up of "Success" at 27 Days

Drug	Cases originally treated	Success at 27 days		Failures/cases presenting		
		No.	%	1 month after discharge	2 months after discharge	3 months after discharge
Tetracycline Diiodohydroxyquinoline Chloroquine	50	47	94	4/32	3/18	0/12
Diiodohydroxyquinoline Chloroquine	25	5	20	1/3	0/2	1/1
Tetracycline Diiodohydroxyquinoline	25	15	60	2/4	—	—
Tetracycline Chloroquine	25	12	48	1/5	0/1	0/1

These results suggested that the reason for the incomplete efficacy of the tetracyclines might lie in their lack of activity against amebas separated from their bacterial symbionts and, in low dosage, against bowel amebas in the cyst-producing phase. Therefore adjuvant therapy was indicated. Chloroquine, though not so effective as emetine in this respect,¹⁰ was indicated against visceral amebas, and a relatively insoluble quinoline, such as diiodohydroxyquinoline, against the cyst-producing amebas.

Tetracycline was used in low dosage, partly because it is difficult to evaluate the effect of adjuvant therapy when the primary drug is already giving a success rate of more than 90 per cent and partly because we were seeking a cheap form of therapy. Various combinations were tried, with the results shown in table II. The prominent feature¹¹ of this series of tests was that when chloroquine alone or diiodohydroxyquinoline alone was used as adjuvant, there was little significant effect, but when both were used together with the low-dosage tetracycline, the result obtained was just as good as with the high-dosage tetracycline by itself. As with low-dosage tetracycline, the rate of healing was slow, more than 70 per cent of cases still showing open ulceration at the tenth day of treatment, but whereas chloroquine and diiodohydroxyquinoline show a success rate at 27 days of only 20 per cent, the addition of tetracycline in the low dosage of 150 mg./day for 10 days raised this rate to 94 per cent. Follow-up is still in progress; the results at the date of writing are shown in table II.

Demethylchlortetracycline,* a new antibiotic, was given to 12 patients in a dosage of 150 mg. four times a day for 10 days. Of these, all showed open ulcers at completion of therapy at 10 days. In 2 patients, parasites were found and change of therapy was mandatory. At the fifteenth day 2 more showed parasites and they too had to be put on other treatment. On the assessment at 27 days, 4 cases were classed as successes, and 2 of these patients are still clear, one month after discharge. The total failure rate was thus 67 per cent—a poor result.

The failure of demethylchlortetracycline by comparison with the other tetra-

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for demethylchlortetracycline is Declomycin.

cyclines is of interest. It may be due to a change of antibacterial spectrum, to a low dosage, or to the rapid absorption¹² leading to a high blood level. The efficacy of the tetracyclines is probably in part due to the width of the spectrum and may follow on their action on some as yet unidentified species of bacteria; a subtle change in the configuration of the tetracycline might preclude this effect. The lower dosage, while it might reduce the efficacy, does not explain everything, for other tetracyclines in lower dosage give better results. A high blood level is of little value in amebic colitis, as is evidenced by our 40 per cent failure rate with intramuscular tetracycline.⁴ Possibly a combination of these factors is operating.

CONCLUSIONS

The activity of the tetracyclines against the ameba is probably in the main indirect; the drugs operate through the bacterial flora that are supporting the ameba in both its commensal and invasive phases. When the ameba has left the immediate area of the bowel, it is apparently able to derive the essential metabolite from the human host. In such a situation, the antibacterial approach is of little or no value. We do not know which species of bacteria or enterovirus can provide the metabolite in the bowel nor whether different organisms or metabolites are necessary for the commensal and invasive phases of *E. histolytica*. It is, however, apparent that the antibacterial approach is inadequate for all phases of amebiasis and that adjuvant therapy is indicated.

Even with smaller dosages of tetracycline, the administration of chloroquine and diiodohydroxyquinoline has a potentiating effect, and the best therapy would probably be to use the tetracyclines at 1 Gm./day, together with routine treatment by either emetine or chloroquine and an efficient luminal amebicide.

SUMMARY

Though the place of the tetracyclines in the treatment of acute amebic colitis is well established, their use has not become general. The application of such therapy to conditions in which the relationship between the symptoms and the ameba is not well established is likely to discredit useful forms of treatment.

More than 300 patients with acute dysentery have been given various tetracyclines, in differing dosages, singly and in combination with other drugs, and the results are compared and contrasted with other forms of therapy. The choice of patients for trial therapy was strict and the assessment was based not only on the disappearance of parasites but also on the sigmoidoscopic appearance from day to day.

As single drugs, some forms of tetracycline are unequaled in the treatment of the acute condition, but the occurrence of relapses and of liver abscess indicates the necessity for adjuvant therapy. With the use of quinoline, smaller dosages of tetracycline may give similarly good results, with protection against complications.

A new antibiotic, demethylchlortetracycline, is shown to be relatively ineffective in these cases.

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Relationship of Drug Resistance to the Production of Urease and Citrase by Some Coliform Bacilli: A Preliminary Report

HARRY SENECA, JOHN K. LATTIMER, MYRON S. ROBERTS, O. K. TROC,
P. MILNER, AND P. PEER

*Department of Urology, Columbia University College of Physicians and Surgeons,
and Squier Urological Clinic, Columbia-Presbyterian Medical Center,
New York, N. Y.*

In a previous report from our laboratories,¹ for the study of urinary infection, gram-negative rods pathogenic to man were classified into six groups, according to their ability to split urea and utilize citrate. In group I, urea is split under both aerobic and anaerobic conditions and the carbon of citrate is utilized in the synthetic medium where citric structure is the sole source of carbon. Most of the clinical strains isolated from our patients fell into this group, namely, *Proteus mirabilis*, *Proteus vulgaris*, and *Proteus rettgeri* and *Proteus* OX 2. Tentatively, we have referred to this group as *Proteus columbiensis*. In group II, urea is split under aerobic conditions, and carbon from the citrate structure is utilized. This group includes coliform bacteria of the types *Aerobacter* and *Klebsiella*. Group III splits urea, but does not utilize the carbon of citrate, and includes *Proteus morganii*. Group IV splits urea under aerobic and anaerobic conditions, but fails to utilize carbon of citrate structure. *Proteus* OX 19 belongs to this group. Group V fails to split urea, but utilizes carbon of citrate structure and includes *Pseudomonas*, *Alcaligenes*, *Vibrion cholerae*, and *Salmonella* genus. Group VI fails to split urea and utilize carbon of citrate structure and comprises *Escherichia*, *Eberthella*, *Shigella*, *Vibrion* El Tor and *Proteus* OX K.

Recently, we isolated a few strains of *Proteus inconstans*, thus adding another group. Group I is *P. columbiensis*; group II is *P. morganii*; group III is *Aerobacter* and *Klebsiella*; group IV is *Proteus* X organisms; group V is *P. inconstans*; group VI includes *Pseudomonas*, *Alcaligenes*, *Salmonella*, *Vibrion cholerae*; group VII includes *Escherichia*, *Eberthella*, *Shigella*, and *Vibrion* El Tor. *P. morganii* is shifted to group II because it splits urea aerobically and anaerobically while *Aerobacter* and *Klebsiella* group III splits urea under aerobic conditions. *P. inconstans* does not split urea, carbon of citrate is utilized in Simmon's citrate and synthetic urea citrate medium.²

Clinically speaking, *Proteus*, *Klebsiella*, *Aerobacter*, and *Pseudomonas* infections are resistant to chemotherapy.^{3,4} Chloramphenicol is the most effective drug against all four gram-negative rods in our experience. A year ago, we observed that kanamycin was very effective in *Aerobacter* infections,⁵ but apparently it is losing some of its initial effectiveness against this mutagenic pathogen. Occasionally these organisms prove to be susceptible to some one drug such as tetracycline, sulfonamide, novobiocin, nitrofurantoin, mandelamine, but sooner or later they develop resistance, although about 50 per cent are still sensitive to chloramphenicol initially. All four of these pathogens split urea, except *Pseudomonas*. On the other hand, most strains of *Escherichia coli* isolated from infections of the urinary tract are susceptible to chemotherapeutic agents.⁶ It was reasoned that the frequent presence of

enzymatic activity (urease and citrase) might imply a link with mutagenicity, and thus with the more rapid acquisition of drug resistance. Accordingly, we studied the enzyme profiles of all the clinical strains of bacteria isolated from our patients, and checked susceptibility patterns to therapeutic agents. As of the writing of this manuscript, we have completed the study of the enzyme profile of the gram-positive cocci.

The present studies include the relationship between urease and citrase activity, and the pattern of drug susceptibility.

MATERIALS AND METHODS

Media for testing urea splitting and utilization of carbon of citrate included Bacto urea medium (Difco), Simmon's citrate agar (Difco), Synthetic urea citrate agar, synthetic citrate agar, and synthetic urea agar.²

Sensitivity patterns of coliform bacteria were determined by the tube dilution method where the antibiotic or antibacterial was serially diluted in brain-heart infusion broth in sterile Kahn tubes in 0.5 ml. amounts. The inoculum, an over-night old culture of either standard, or clinical strain, grown in brain-heart infusion broth, was diluted 10^{-3} . In the case of sulfonamide it was diluted 10^{-10} . One-half ml. was then added to the tubes containing the drugs, so that the final concentration of all drugs ranged from 100 $\mu\text{g./ml.}$ to 0.19 $\mu\text{g./ml.}$, and in the case of sulfonamide it ranged from 5 to 0.037 mg./ml. The final concentration of penicillin-streptomycin combination ranged from 50 units + 50 $\mu\text{g./ml.}$ to 0.09 units + 0.09 $\mu\text{g./ml.}$

Strains used in these studies included: (1) Standard strains of *Aerobacter aerogenes* (Reilly, 271, 189, 79, 76 and Dobriner); *Klebsiella pneumoniae* (10031 FDA, Types I, II, III U. S. Army; *E. coli* (Pfizer, 017, 055, 0126 U. S. Army, and Turkey strain U. S. Navy). (2) In one study in 1959 in which demethylchlor-tetracycline, tetracycline, and sulfadiazine were studied, 16 recent clinical strains of *E. coli* and one strain of *K. pneumoniae* were included. (3) From a previous study of the over-all sensitivity pattern, 30 clinical strains of *E. coli*, 30 clinical strains of *A. aerogenes*, and 3 strains of *K. pneumoniae* were compared as to the inhibitory concentration of nitrofurantoin, tetracycline, chloramphenicol, erythromycin, kanamycin, novobiocin, penicillin-streptomycin, streptomycin, and sulfadiazine.

RESULTS

All strains of *A. aerogenes* and *K. pneumoniae* tested, split urea under aerobic conditions as manifested by the change of color of the slant to red of Bacto urea (Difco), synthetic urea citrate, and synthetic urea media. Carbon of the citrate structure was utilized since there was growth and change of color of the slant of Simmon's citrate from green to blue, and growth on synthetic citrate medium. Thus, both showed evidence of urease and citrase activity.

E. coli neither split urea nor utilized carbon from citrate structure in synthetic media, and thus failed to show evidence of urease or citrase activity.

Study 1. Susceptibility pattern of standard strains. Five strains of *A. aerogenes* were checked against five drugs. The minimum inhibitory concentration of nitrofurantoin, tetracycline, and erythromycin was 100 $\mu\text{g./ml.}$; sulfadiazine 5 mg./ml.;

TABLE I

Sensitivity Pattern of 16 Clinical Strains of E. coli and 42 Clinical Strains of A. aerogenes to Tetracycline, Demethylchlortetracycline, and Sulfadiazine

	<i>E. coli</i> (16)		<i>A. aerogenes</i> (42)		<i>K. pneumoniae</i>
	12.5-100	.19-6.25	12.5-100	.19-6.25	12.5-100
Tetracycline	7	9	39	3	1
Demethylchlortetracycline	7	9	37	5	1
Sulfadiazine	12	4	42	—	1

chloramphenicol 50 µg./ml., two strains, 25 µg./ml., two strains, and 6.25 µg./ml., one strain; penicillin plus streptomycin 50 units plus 50 µg./ml.

Five strains of *E. coli* showed minimum inhibitory concentration for nitrofurantoin of 12.5 to 50 µg./ml.; tetracycline 0.39 to 0.78 µg./ml.; chloramphenicol 0.78 to 6.25 µg./ml.; erythromycin 25 to 100 µg./ml.; penicillin-streptomycin 6.25 + 6.25 to 25 units + 25 µg./ml.; sulfadiazine, 0.62 to 5 mg./ml.

Four strains of *K. pneumoniae* showed minimum inhibitory concentration for nitrofurantoin, 6.25 to 50 µg./ml.; sulfadiazine, 0.019 to 0.78 mg./ml.; tetracycline, 0.19 to 3.125 µg./ml.; chloramphenicol, 0.39 to 3.12 µg./ml.; erythromycin, 1.56 to 25 µg./ml.; penicillin-streptomycin, 0.19 + 0.19 to 0.78 units + 0.78 µg./ml.

Study 2. Table I shows the sensitivity pattern of the 16 strains of *E. coli*, 42 strains of *A. aerogenes*, and 1 strain of *K. pneumoniae* from clinical cases, to tetracycline, demethylchlortetracycline, and sulfadiazine. Nine strains of *E. coli* were sensitive to tetracycline, 9 to demethylchlortetracycline, and 4 to sulfadiazine. On the other hand, 3 strains of *A. aerogenes* were sensitive to tetracycline, 5 to demethylchlortetracycline, and none to sulfadiazine. One strain of *Klebsiella* was resistant to all three drugs.

Study 3. Table II shows the sensitivity pattern of 30 clinical strains of *E. coli*, 30 clinical strains of *A. aerogenes*, and 3 clinical strains of *K. pneumoniae* to nitrofurantoin, tetracycline, chloramphenicol, erythromycin, kanamycin, novobiocin,

TABLE II

Sensitivity Pattern of 30 Clinical Strains of Escherichia and 30 Clinical Strains of Aerobacter and 3 Clinical Strains of Klebsiella to Nitrofurantoin, Tetracycline, Chloramphenicol, Erythromycin, Kanamycin, Novobiocin, Penicillin-streptomycin, Streptomycin, and Sulfadiazine

	<i>E. coli</i> (30)		<i>A. aerogenes</i> (42)		<i>K. pneumoniae</i>
	12.5-100*	.19-6.25	12.5-100	.19-6.25	12.5-100
Nitrofurantoin	26	4	30	—	3
Tetracycline	19	11	27	3	3
Chloramphenicol	4	26	14	16	3
Erythromycin	28	2	30	—	3
Kanamycin	2	3	2	10	3
Novobiocin	25	1	29	1	3
Penicillin-streptomycin	22	1	25	—	3
Streptomycin	25	1	19	—	3
Sulfadiazine	27	3	28	2	3

* Minimum inhibitory concentration expressed in µg./ml.

penicillin-streptomycin, streptomycin, and sulfadiazine. Four *E. coli* are sensitive to nitrofurantoin, 11 to tetracycline, 26 to chloramphenicol, 2 to erythromycin, 3 to kanamycin, 1 to novobiocin, 1 to penicillin-streptomycin, 1 to streptomycin, and 3 to sulfadiazine. None of the 30 *A. aerogenes* are sensitive to nitrofurantoin, 3 are sensitive to tetracycline, 16 to chloramphenicol, none to erythromycin, 10 to kanamycin, 1 to novobiocin, none to penicillin-streptomycin and streptomycin, and 2 to sulfadiazine.

None of 3 clinical strains of *K. pneumoniae* were sensitive to these nine drugs.

DISCUSSION

All *A. aerogenes* strains tested produced evidence of urease and citrase activity manifested by the splitting of urea in media, and utilization of urea where the sole source of carbon was urea, and also utilized carbon from the citrate structure where the latter was the sole source of carbon. *K. pneumoniae* showed the same enzyme profile as *A. aerogenes*. On the other hand, *E. coli* was completely devoid of activity by these two enzyme systems.

The close correlation between enzyme profile and pattern of drug resistance was interesting. Previously, we had shown that *A. aerogenes* mutated readily to resistant forms while *E. coli* did not, (in relation to tetracycline). At the present, it is not possible to find an explanation for this variation in the capacity for mutation in this coli-aerogenes group.

Standard laboratory cultures of *A. aerogenes* are very frequently resistant to antibacterials and antibiotics, while *K. pneumoniae* tend to be rather sensitive, although they both have the same enzyme profile. Possibly the reason is that these laboratory strains of *Klebsiella* have not yet been subjected or exposed to drug action.

This same high resistance was also observed in strains of *A. aerogenes* obtained from clinical cases. They were resistant to tetracycline, demethylchlortetracycline, erythromycin, novobiocin, penicillin-streptomycin, streptomycin, sulfadiazine, and nitrofurantoin. On the other hand, they were rather sensitive to chloramphenicol and kanamycin. This observation involved 72 strains of *A. aerogenes*. All four strains of *K. pneumoniae* obtained from patients who had been exposed to chemotherapy were resistant to all drugs. This was in marked contrast to the findings in laboratory strains of *K. pneumoniae*, which were sensitive.

In the case of clinical strains of *E. coli*, the general tendency was toward continued drug susceptibility. They were quite susceptible to tetracycline, demethylchlortetracycline, chloramphenicol, kanamycin, and there were more additional strains sensitive to sulfadiazine, nitrofurantoin, erythromycin, novobiocin, penicillin-streptomycin, and streptomycin than were found among *A. aerogenes*.

Since capacity for mutation and enzyme production are both related to the desoxyribonucleic acid in the cell structure of an organism, it may be feasible to alter capacity for mutation, enzyme profile, and desoxyribonucleic acid. Already, we⁷ have been able to block urease activity by *Bacillus proteus* and thus alter the susceptibility pattern from "drug resistance" to "susceptibility" by the action of an enzyme blocking agent, chlormerodrin. This had been previously demonstrated in

vitro,⁸ and clinical trials are in progress to test the efficacy of blocking mechanism against urease production, in patients who are either suffering of calculus disease of the kidney or chronic urinary tract infections caused by urea splitting organisms. If effective, this may herald an important step forward toward a new method for the treatment of resistant urinary infections.

SUMMARY

1. *Aerobacter* and *Klebsiella* split urea and utilize carbon of the citrate structure. Both clinical and standard laboratory strains of *A. aerogenes* are resistant to antibiotics and antibacterials, while laboratory strains of *Klebsiella* are still rather susceptible. However, both the clinical strains of *Aerobacter* and *Klebsiella* are resistant to these drugs.

2. *E. coli* fails to split urea or to utilize the carbon of the citrate structure. The standard laboratory strains are very sensitive to antibacterials and antibiotics. Most of the clinical strains recovered from hospital and/or clinic patients are still sensitive to drugs.

3. It would appear that the organisms which demonstrated the most enzymatic activity (urease and citrase, in this case) had the greatest ability to mutate to resistant forms.

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In Vitro Study of the Action of a Tetracycline-Oleandomycin Preparation, Tetracycline, Oleandomycin, and Chloramphenicol on *Staphylococcus*, *Escherichia coli*, and *Klebsiella*

RAUL YAZIGI, JORGE VIGOUROUX, AND YOLANDA MORENO

University of Chile and San Juan de Dios Hospital, National Health Service, Santiago, Chile

The action of combinations of antibiotics against bacteria and the development of sensitivity to various antibiotics constitute two problems of great interest to clinicians as well as bacteriologists.

The synergistic action of penicillin and sulfonamide drugs was studied by Ungar,¹ Bigger,² and Vigouroux and Leyton^{3,4} as early as 1943. Vigouroux and Leyton demonstrated that the synergism of penicillin and the different sulfonamide drugs varied for each strain of bacteria. They proved, furthermore, that in a combination of two antibiotics one of the two components could be absolutely inactive against a bacterium when tested alone, but the synergistic action appeared nevertheless, and it might attain a high intensity. The same phenomenon was demonstrated by Jawetz and Gunnison⁵ using a penicillin-streptomycin combination acting on enterococci. There followed then a series of publications dealing with the synergistic action of antibiotics, including those of Vigouroux and Ebenspurger,⁶ Welch,⁷ and Lamansen.⁸ Lamansen has observed in vivo the existence of synergism in various combinations of antibiotics, including those that formerly were considered unwanted as in the case of chlortetracycline and penicillin. The instances of antagonism observed at times in vitro may be debatable, inasmuch as the in vitro test represents the administration of one single dose of the antibiotic. This is never the case in clinical therapy.⁹

The emergence of bacterial resistance to various antibiotics represents a serious future problem, and in fact, it has already caused difficulties for the clinician. The selection of resistant bacteria or their mutations is a problem that has not been solved as yet, particularly in the closed environments of hospitals. Nevertheless, many authors, including Klein and Schorr¹⁰ and others, have performed experiments demonstrating that combinations of antibiotics can inhibit, partly or entirely, the development of bacterial resistance. The existence of synergism has already been demonstrated although we do not know precisely how it is produced. Consequently, one obvious procedure in the fight against the emergence of bacterial resistance, which is precisely our present problem, is the use of combinations of antibiotics.

Our study has been carried out in two parts. The first part consists of a comparative study of bacterial inhibition using discs impregnated with various antibiotics placed on solid culture media seeded with different test organisms. The second part consists of a similar study but carried out using the twofold serial tube dilution technique, which will be reported later. We have decided to do our study in this double fashion because the disc or tablet technique in solid media has been criticized by some authors as inadequate for precise comparison of differ-

ent antibiotics. This is particularly true of combinations of antibiotics when differences in rates of diffusion, differences in molecular size, and other factors complicate the picture. However, in a previous study of 100 strains of *Shigella* there was no significant difference between the results of the disc method and the tube dilution method.

We have also compared the bactericidal activity of oleandomycin and tetracycline, alone and in combination, against several strains of *Micrococcus pyogenes* var. *aureus*, using Chabbert's modification of the classical serial tube dilution technique. The latter studies are not complete but preliminary results confirm the findings reported here.

This present preliminary report summarizes the results of studies on the bacteriostatic activity of the antibiotics measured by means of standardized disc technique.

MATERIAL AND METHODS

The bacteria selected for the present study consisted of 203 recent clinical isolates. There were 100 strains of *M. pyogenes* var. *aureus*, of which 54 were isolated from acute suppurative infections and 46 from pharyngeal secretions of healthy persons. There were 51 strains of *Escherichia coli* isolated from acute urinary infections. The remainder consisted of 52 strains of *Klebsiella* of various origins.

Tetracycline and oleandomycin were studied individually and compared to a mixture of the two containing two parts of tetracycline to one part of oleandomycin.

RESULTS

Staphylococci from Acute Infections. The 2:1 mixture of tetracycline and oleandomycin proved to be superior, on a weight for weight basis, to tetracycline alone in 78 per cent of the strains tested. The activity of the mixture was equal to that of tetracycline alone in 15 per cent of the strains tested. In the latter situation it must be emphasized that the mixture contained only two thirds of the amount by weight which was present in the agar containing tetracycline alone. Thus, in 93 per cent of the strains tested synergism was demonstrated for tetracycline.

In a similar manner, the mixture showed superior activity to oleandomycin alone in 57 per cent of the strains tested. It was equal (although containing only one third of the amount of oleandomycin) in 22 per cent of the strains. Thus, synergism was demonstrated for oleandomycin in 79 per cent of the strains isolated from acute suppurative lesions.

Staphylococci from Pharyngeal Secretions of Healthy Persons. The tetracycline-oleandomycin mixture was found to be equal to or superior to tetracycline alone in 74 per cent of the strains tested. It was equal to or superior to oleandomycin alone in 78 per cent of the strains.

Escherichia coli Strains. The tetracycline-oleandomycin mixture was found to be equal to or superior to tetracycline alone in 76 per cent of the strains and superior to oleandomycin alone in 96 per cent of the strains of *E. coli* tested.

Klebsiella Strains. The mixture of tetracycline and oleandomycin was equal to or

superior to tetracycline alone in 76 per cent, and superior to oleandomycin in 100 per cent of the *Klebsiella* tested.

DISCUSSION

In examining the current situation of the sensitivity of bacteria to various antibiotics we frequently find a high resistance to several antibiotics at the same time, and this circumstance suggests immediately that it is advisable to make use of combinations of antibiotics.

In a study of the progressive resistance to antibiotics observed in bacteria isolated in Paris in the years 1949 to 1954, Chabbert et al¹² demonstrated that staphylococci acquire a distinct resistance to penicillin. These findings have been confirmed by many others who have established that this resistance might extend to more than 80 per cent of the strains.

When a clinical case involves an infectious process caused by a pathogenic organism resistant to every available antibiotic, there still remains a chance for success, by using a suitable combination. Chabbert¹³ states: "One of the most interesting effects of the combinations of antibiotics is precisely the increase in the speed of the bactericidal action and, consequently, the reduction of the number of surviving bacteria within a given period of time."

The studies of Lamansen in the Pasteur Institute demonstrated that the combination of antibiotics yielded the best results when the antibiotics were administered simultaneously, whereas the results were bad when they were administered at different times. Bunn et al¹⁴ proved that certain antibiotic combinations could produce a synergistic effect even with a bacterium as highly resistant as *Pseudomonas aeruginosa*. Winton and Chesrow¹⁵ proved that the tetracycline-oleandomycin preparation was able to control 96 per cent of clinical cases of mixed infections.

In our study, the tetracycline-oleandomycin mixture demonstrated superior antibacterial activity over its individual components in the majority of bacteria studied.

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Blood Level Studies with a New, Preconstituted, Intramuscular Solution of Oxytetracycline

RAMON NARANJO J.

Department of Biochemistry, School of Medicine, University of Guadalajara, Guadalajara, Jal., Mexico

The molecular structure of oxytetracycline is enolic and therefore its combination with bivalent metallic ions, such as magnesium, manganese, iron, copper, nickel, copper, and zinc, is easily produced; these combinations are insoluble in water but they are soluble in some organic solvents.¹⁻⁴

These facts establish the possibility that these metallic compounds, due to their stability, may be appropriate for obtaining high and constant blood levels of antibiotics. Hammer et al.⁵ demonstrated the solubility and stability of these compounds in organic solvents of aqueous N,N-dimethylacetamide type.

We felt that it would be interesting to determine the blood levels of a preconstituted intramuscular solution consisting of an 80 per cent propylene glycol vehicle containing 1 to 1 molar ratios of oxytetracycline base to magnesium chloride, with lidocaine as the anesthesia and later comparing these results with those obtained with intramuscular administration of 100 mg. of a dry-fill preparation of oxytetracycline to which a solvent is added prior to injection.

MATERIAL AND METHODS

A preconstituted intramuscular solution of oxytetracycline containing 125 mg./ml. was injected into 10 normal human subjects. Each subject received an intragluteal injection of the contents of 1 ampule (2 ml.) at 0, 24, and 48 hours. Ten ml. blood specimens were taken from each subject 4, 12, 24, 36, 48, 60, 72, 84, and 96 hours thereafter and tested for biological activity. The assay method for oxytetracycline in blood specimens is described by Grove and Randall.⁶ Subsequently, another group of human subjects was injected with the dry powder formulation of oxytetracycline intramuscular containing 50 mg./ml. of antibiotic hydrochloride when diluted for injection. Ten subjects received intramuscular injections of the contents of 1 ampule (2 ml.) at 0, 12, 24, 36, 48, and 60 hours and blood samples were taken at intervals similar to those in the other series.

RESULTS AND DISCUSSION

Blood samples taken from persons given multiple doses of preconstituted intramuscular solution of oxytetracycline (250 mg./2 ml.) and dry-fill solution (100 mg./2 ml.) are given in tables I and II.

It has been demonstrated that antibiotics, including oxytetracycline, accumulate in the liver.⁷ These data form the basis of some speculations regarding the relationship between the liver and blood levels and show the convenience of using a pre-

TABLE I

Serum Levels, $\mu\text{g./ml.}$, Multiple Injections in Human Beings of Preconstituted, Intramuscular Solution of Oxytetracycline, 250 mg. every 24 hours. Lot no. 91-438-53 EPD

	Sample taken, hour intervals*								
	4	12	24	36	48	60	72	84	96
1.	2.080	.960	.760	1.400	1.200	1.000	.600	.380	.060
2.	3.320	1.440	.600	.840	1.760	1.160	.500	.300	.121
3.	2.520	0.960	.480	1.040	.920	.920	.400	.240	.100
4.	1.120	.920	.480	.800	1.560	1.400	.600	.120	.020
5.	.920	.600	.480	.680	1.120	.920	.520	.280	.180
6.	.800	.800	.320	.920	.920	.800	.480	.100	.080
7.	.920	.680	.480	.680	.800	.680	.400	.180	.050
8.	.920	.800	.600	1.120	1.400	.920	.600	.260	.120
9.	1.040	.560	.200	1.400	1.040	1.600	1.480	.800	.040
10.	1.480	.800	.200	.840	1.040	1.120	.720	.400	.120
Average $\mu\text{g./ml.}$	1.512	.852	.460	.972	1.176	1.056	.630	.306	.089

* Injection at 0, 24, and 48 hours.

constituted antibiotic formulation that will be adequately absorbed at the site of the injection during an extended period. Although quantitative results on microbial sensitivity cannot be directly related to antibiotic serum levels, they can be a useful basis for guiding judgment in the clinic. The data previously established⁸ and the results obtained show that therapeutic blood levels of oxytetracycline are obtained with the new preconstituted intramuscular solution of oxytetracycline.

TABLE II

Serum Levels, $\mu\text{g./ml.}$, Multiple Injection in Human Beings of Oxytetracycline Intramuscular Solution, Dry-fill Preparation, 100 mg. every 12 Hours

	Sample taken, hour intervals*									
	4	12	24	36	48	60	72	84	96	108
1.	1.200	.600	1.300	.800	1.720	1.880	1.380	.640	.180	.040
2.	1.520	.600	1.960	.920	1.720	2.200	1.360	.520	.080	.008
3.	1.040	.600	.800	1.160	1.200	1.720	1.080	.520	.180	.005
4.	1.040	.720	1.160	1.040	1.600	1.520	.920	.400	.040	.004
5.	.600	1.160	1.280	2.640	2.600	1.560	.800	.600	.260	.100
6.	1.720	1.120	1.840	2.200	1.040	1.520	1.920	.600	.100	.080
7.	1.160	.600	1.000	.680	1.520	1.160	1.040	.400	.140	.040
8.	1.040	.600	.800	1.720	1.480	1.120	.920	.560	.200	.020
9.	1.160	.800	1.420	1.840	1.400	1.040	.680	.400	.140	.010
10.	1.000	.680	.720	1.400	1.840	1.520	1.040	.400	.140	.040
Average $\mu\text{g./ml.}$	1.148	.748	1.228	1.440	1.612	1.524	1.114	.504	.136	.034

* Injections at 0, 12, 24, 36, 48, and 60 hours.

SUMMARY

In the normal human subjects who were used in this study, the following conclusions can be appropriately drawn.

1. The average blood levels obtained after 24 hours with one injection of 250 mg./2 ml. of preconstituted intramuscular oxytetracycline can be considered therapeutic.

2. Adequate serum concentrations of oxytetracycline after a multiple intramuscular dose of a new preconstituted solution were demonstrated at 24, 48, and 72 hours (average 0.460, 1.176, and 0.630 $\mu\text{g./ml.}$ respectively) in 10 normal human subjects.

3. The blood levels after 96 hours with three injections of preconstituted intramuscular oxytetracycline of 250 mg. every 24 hours are higher and longer lasting than those obtained with six injections of dry-fill oxytetracycline, 100 mg. administered at 12 hour intervals.

4. This report should be considered as preliminary until the results relative to the comparative tolerance of both preparations can be analyzed.

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An Experimental Study of Oxytetracycline: Local Genital Absorption and Diffusion, and Placental Transmission to the Blood of the Umbilical Cord and to the Amniotic Fluid

DEOBALDO FEBLES-ALFONZO* AND CARLOS BATTHYANY†

*Gynecological and Obstetrical Clinic and the Family Allowance
Medical Social Service, Montevideo, Uruguay*

In the prevention and treatment of infections of the genital organs, different methods of administering antibiotics have been sought.

Some years ago Crottogini was already practicing direct intrauterine administration of sulfathiazole with an apparatus he designed himself. Local antibiotic therapy in gynecology and obstetrics has been a constant aim with us. In 1947 Crottogini and Giampietro³ were engaged in the study of local penicillin therapy in gynecology.

Nowadays the development of new antibiotics has made it necessary to return to the study of local administration of antibiotics, since the field of application is wide.

For this reason we have started a series of experiments to study the absorption of antibiotics administered intrauterinely.

It must be borne in mind that the absorption capacity by this means of administration causes the antibiotic to act in a double capacity, i.e., locally and by absorption.

We completed this study with a series of patients in whom the placental transmission of antibiotics was investigated, measuring concentration in the blood of the umbilical cord and in the amniotic fluid.

In all the experiments oxytetracycline‡ was used.

MATERIAL AND METHODS

We have studied 25 patients, 16 of whom were in labor, the others having various gynecological diseases.

In the first group we studied placental transmission of oxytetracycline, and in the second, intrauterine absorption of this antibiotic.

To study placental transmission, a solution of oxytetracycline in concentrations varying between 0.5 and 1.56 per cent was given by intravenous injection to the mother during the course of labor. The total amount of antibiotic injected varied from 125 to 500 mg. In 1 case (patient no. 3) the method of oral administration was used.

Later, at varying intervals, samples of the amniotic fluid were extracted by puncture of the membranes with sterilized equipment. At the moment of birth a sample of blood was taken from the umbilical cord, and immediately afterward a sample of the mother's blood was taken by venous puncture.

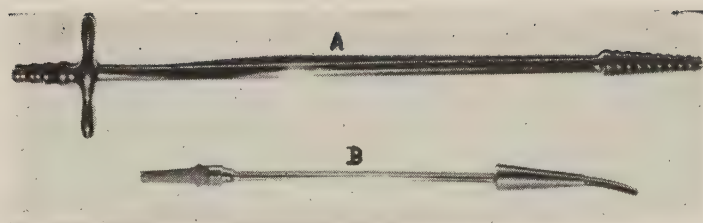
For the study of intrauterine absorption a technique similar to that employed in hysterosalpingography was used. In our clinic, the catheter of Risolía, modified

* Doctor of Professor Crottogini's Clinic.

† Bacteriologist, Dispert Laboratory.

‡ The trade name of Chas. Pfizer & Co. for oxytetracycline is Terramycin.

FIG. 1. Catheters used in our investigation: A. Catheter of Risolia, modified by Parada; B. Thinner catheter especially designed by us.



by Parada, was used (fig. 1, A). In addition to this catheter we have had made a smaller and thinner one, which has the advantage that it passes through the internal orifice of the cervical canal (fig. 1, B).

The quantities of oxytetracycline injected varied between 50 and 125 mg. Later, blood samples were taken by venous puncture one, four, and eight hours after the intrauterine injection.

All studies of antibiotic levels were carried out according to the technique described by Grove and Randall, by the dilution method. Oxytetracycline of the Antibiotic Diagnostic Kit was used as the standard. The organism used was *Micrococcus pyogenes* var. *aureus* from the collection (C 127), which was sensitive to 0.8 mg. of oxytetracycline in stable form.

The amniotic fluid and the serum of the blood of the umbilical cord were sterilized by Seitz filter.

RESULTS

Local Intrauterine Absorption. Table I shows that with 50 mg. oxytetracycline administered to 7 patients, and 125 mg., to other 2, the results in blood levels varied greatly, which would indicate that there are individual factors, general or local, that prevent a greater diffusion of the antibiotic or provoke destruction in situ. Although it is true that the small number of persons treated with 125 mg. prevent us from drawing exact conclusions, we may nevertheless conclude that the useful dosages of this drug as already mentioned, must vary in accordance with the patient or disease.

In our studies, after intrauterine injection, blood levels showed a rather high peak after two hours but decreased until there was no trace of the drug at the fifth or sixth hour.

TABLE I
Results of Local Intrauterine Administration

No. of cases	First hour, µg./ml.	Fourth hour, µg./ml.	Eighth hour, µg./ml.
1	25	6.3	0.8
2	12.5	0.8	0.0
3	6.3	0	0
4	3.1	0.8	0
5	0	0	0
6	12.5	6.3	0
7	6.3	0	0
8	12.5	0.8	0
9	1.6	0	0

TABLE II
Placental Transmission

Patient	Amount injected	Level in amniotic fluid, μg./ml.	Level in cord, μg./ml.	Level in mother's blood, μg./ml.
1	125 mg. intravenously	—	0	—
2	125 mg. intravenously	0	0	—
3	1 Gm. orally	0.8	3.1	—
4	250 mg. intravenously	0	0	0
5	250 mg. intravenously	1.6	1.6	3.1
6	250 mg. intravenously	0	3.1	6.2
7	250 mg. intravenously	0	3.1	12.5
8	250 mg. intravenously	0	0	0
9	250 mg. intravenously	0	6.3	12.5
10	250 mg. intravenously	0	6.3	12.5
11	250 mg. intravenously	0	3.1	6.3
12	250 mg. intravenously	0	3.1	6.3
13	250 mg. intravenously	0	3.1	12.5
14	250 mg. intravenously	0	0.8	3.1
15	250 mg. intravenously	0	3.1	12.5
16	500 mg. intravenously	0	12.6	25

The blood levels obtained apparently do not coincide directly with the total dosage injected. We believe that there must be various anatomical, biological, or functional factors that have a great influence on the absorption of the antibiotic.

Permeability or tubal obstruction has great importance from the anatomical point of view. Tubal permeability in effect permits the passage of the antibiotic solution into the peritoneum with the added advantage of its absorption.

Placental Transmission. Table II shows transmission of the antibiotic into the fetal circulation. A direct relation between levels in the fetal circulation and the dosage administered to the mother can be observed. Although with 150 mg. administered intravenously, no traces of the antibiotic were found in the fetus, when the dose was increased to 250 mg. or more, the antibiotic was found in the fetal blood.

The drug was found in the amniotic fluid of only 2 of 16 cases. This proves that passage of the drug into the amniotic fluid would be much more evident with higher dosages.

Our studies were made immediately after extraction of the amniotic fluid, and we found that oxytetracycline is more unstable in this fluid than in any other part of the body. Thus rapid destruction could be the main cause of negative results obtained in the amniotic fluid. Research on the stability of antibiotics in the amniotic fluid is being carried out and will be the subject of a future paper.

TOLERANCE

Tolerance was excellent, no adverse effect having been caused by the action of the drug to either the mother or the fetus.

DISCUSSION

Local administration of antibiotics in gynecology may be undertaken in three

ways (Crottogini and Giampietro³): Strict prophylaxis; as a preventive measure, but only in complicated operative wounds or in certain blood retentions; curative therapy of infections.

Our study is concerned with one aspect of local antibiotic therapy, i.e., intra-uterine administration.

The technique is simple, and this treatment is indicated not only in uterine or endometrial infections, but also in adnexal and particularly tubal inflammations, in treatments for sterility, and in other conditions.

With reference to maternal administration of antibiotics, placental transmission affords a medium of treatment and prophylaxis against the multiple infections that the fetus might acquire in the uterus.

Various investigators^{1,2,7} have studied placental transmission of the sulfonamides. Recently other authors have also studied placental transmission of some of the newer antibiotics. The suitability of this method of administration in the prophylaxis of amniotic infection in premature rupture of the ovular membranes is worth noting.

SUMMARY AND CONCLUSIONS

Twenty-five patients were studied who had been given oxytetracycline, either by intrauterine administration in order to study the absorption capacity, or intravenously to the mother in labor in order to study placental transmission of the antibiotic and its passage into the blood of the umbilical cord and the amniotic fluid.

Local intrauterine administration provides variable but sure absorption of the antibiotic. This probably depends on the peritoneal absorption.

Technique is simple and is similar to the injection of the opaque substance in hysterosalpingography, but with the great advantage of dealing with solutions of much quicker diffusion.

The use of antibiotics in this way, in adequate dosage, will facilitate prophylaxis and treatment of many gynecologic and obstetric infections.

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The Accumulation of Oxytetracycline in Osteogenetic Zones as Measured by Observation of Fluorescence

JOSE PLAZA-ROCA

Department of Traumatology, Central Police Hospital, Lima, Perú

In 1956 we presented to the Peruvian Pathology Society our initial observations on fluorescent microscopy, with particular emphasis on osseous pathology, as well as the systematic use of ultraviolet light or Wood's black light for the gross examination of tissue, particularly specimens of human bone that was obtained in surgery.¹

Shortly thereafter, we broadened our studies to include various fluorescent medicinal substances, such as the acridine derivatives, Mercurochrome, Merthiolate, eosine, sodium salicylate and others; ultimately we employed these materials in the production of various fluorescent paintings, which were shown in an art exhibition.² Among the substances we used was oxytetracycline, which exhibited marked yellow fluorescence, with the peculiarity that aqueous solutions of the substance did not fluoresce whereas the stains left by the solution on filter paper, on which we made our paintings, possessed this property, presenting the same yellow fluorescence observed in dry tetracycline powder. Since we preferred the yellow of fluorescein, we attributed no special merit to this finding.

Subsequently, in studying the fluorescence of bone specimens from osteomyelitic patients we observed an unusual yellow fluorescence, which we at first thought might have been due to the topical application of Merthiolate or Mercurochrome to the fistulas of patients. It was quickly noted, however, that this fluorescence was similar to that we had encountered previously in our experiments with oxytetracycline.

Since then we have broadened and intensified our studies of oxytetracycline fluorescence in osteomyelitis and in this paper we present various findings that indicate that oxytetracycline fluorescence in osteomyelitis is localized principally in osteogenetic regions. We also attempt to outline a therapeutic approach designed to promote the concentration and accumulation of this fluorescent substance in osseous tissue, which approach might be used not only as a prophylactic measure in the osteogenetic regions of affected bones, but also to protect normal spongy bone implants employed in filling areas of osteolysis.

METHODS AND MATERIALS

Bone fragments from patients with chronic osteomyelitis, taken from areas immediately affected as well as from those removed from the focus of infection, were studied. Most of the patients ranged in age from 10 to 12 and 20 to 30 years. We observed fluorescence in the normal spongy bone taken from the iliac crest of these patients; it should be pointed out, as figure 1 shows, that this region has a band of osteogenesis subjacent to the growth cartilage, which is the last region in man

FIG. 1. A. Human bone affected with osteomyelitis showing marked yellow fluorescence in the osteo-genetic region at the focus of infection (shown in black). Yellow fluorescence of lesser intensity in the areas adjacent to the site of infection is indicated by shading.

B. Bone from a rooster showing intense yellow fluorescence in the metaphysis (black), with a lesser degree of fluorescence in the diaphysis (shaded area). C. Human iliac bone showing marked fluorescence subjacent to the cartilage of the crest (black), from which bone graft material is taken. The less intense fluorescence in the area farther removed from the crest (shaded) should be noted.



to complete ossification. All bone specimens were taken from patients who had received oxytetracycline in the usual form for various periods. The bones of some patients still fluoresced even after oxytetracycline therapy had been discontinued for two months.

To roosters approximately 2 months old, we administered oxytetracycline in 250 mg. capsules, forcibly opening the beak whenever required. Some of the fowl were sacrificed after four days of daily administration of this dosage; others received this dosage on alternate days for periods up to six months.

Fresh bone specimens were studied grossly under black light (Wood) in a dark room, using a Phillips type HPW, 125 watt analyzing lamp; some were studied in situ in the operating room.

Specimens were then stored in stainless steel containers or in hermetically sealed glass containers and kept under refrigeration at the temperature normally used in storing food. Samples from each batch of specimens were stored in ethyl alcohol (95 per cent), in which they retain fluorescence for periods up to a year.

We subsequently covered the glass containers with black paper, since we discovered that portions of bone exposed to light, unlike the opposite portions kept in darkness, slowly ceased to fluoresce.

In the study of histological sections with fluorescent microscopy we used ultra-violet light, with an ordinary microscope, the eyepiece provided with an appropriate filter.

The tissue sections fixed in ethyl alcohol (95 per cent) were not decalcified for these studies, since we wished to avoid elimination of fluorescence; for the same reason sections of the soft osteo-genetic areas were obtained by freezing the specimens. Thus, our studies with fluorescent microscopy were limited to this type of tissue.

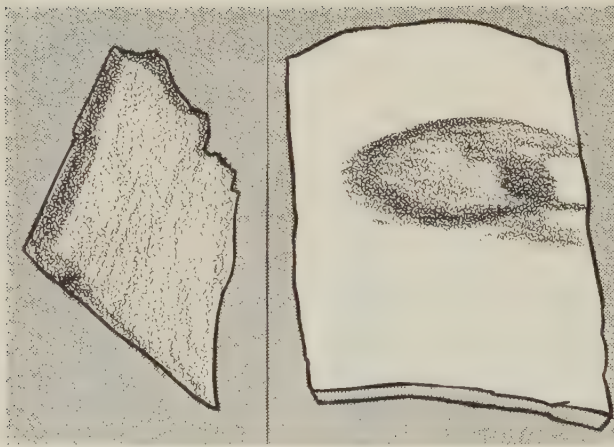


FIG. 2 (left). Filter paper impregnated with oxytetracycline in aqueous solution showing intense yellow fluorescence.

FIG. 3 (right). Fragment from a cast removed from a patient with a fractured leg. Yellow fluorescence of oxytetracycline appears where the exudate from an ulcer has impregnated the cast.

From each of the specimens, we prepared sections fixed in 10 per cent formol solution and decalcified, which were studied with hematoxylin-eosin under the microscope with ordinary lighting. More recently, we began the study of the fluorescence shown by dressings removed from the fistulas of patients receiving oxytetracycline, as well as the plaster casts stained by secretions, all of these presenting a yellow fluorescence similar to that of oxytetracycline (fig. 2).

RESULTS

Fragments from areas directly affected by osteomyelitis showed an intense yellow fluorescence, similar to that of oxytetracycline, but of much greater intensity than that of specimens taken from regions farther removed from the focus of infection, suggesting that fluorescence appears selectively in the osteogenetic regions of infected bone (fig. 1 A).

Fragments of spongy bone removed from the iliac crest to be employed as implants also showed marked fluorescence for this also is a region of normal osteogenesis (fig. 1 C). Sequestrations exhibited no yellow fluorescence, but rather appeared dull white as they normally do.

Osseous fragments from areas affected by osteomyelitis often showed zones of marked fluorescence alternating with zones of slight fluorescence, having the general appearance of leopard's spots; the areas of most intense fluorescence correspond to the osteogenetic regions.

Bones removed from the young roosters exhibited marked fluorescence which tended to grow more intense in the metaphysis (the osteogenetic region) as compared with that of the diaphysis. After a few weeks of the administration of oxytetracycline in the form and dosage indicated, most of the specimens showed generalized fluorescence throughout the bone, with no differentiation in the metaphysis. This result may have been produced by the comparatively high dosage, on the basis of weight, which we administered to the fowl.

As figure 1 reveals, it may be concluded that induced fluorescence in the bones as both human beings and roosters is greater in osteogenetic regions, regardless of whether such areas are normal (as in the growing metaphysis of the chicken and in

the human iliac bone) or pathological (as in the productive areas in cases of osteomyelitis).

We have noted that the bones studied continued to fluoresce for many months (up to one year at this writing), both in the case of the specimens kept under refrigeration, as well as those left in the open or kept immersed in ethyl alcohol. Bones exposed either to open air or to sunlight show appreciable diminution of fluorescence in the areas touched by light, while the parts remaining in darkness retain fluorescence. Similarly, fresh specimens kept moist in refrigerated containers lose fluorescence if subjected to ultraviolet light for long and continuous periods (30 minutes to 1 hour).

Because of this fact we instituted the practice of protecting specimens from light, covering the outside of glass containers with black paper.

DISCUSSION

We have previously suggested that oxytetracycline is localized in osteogenetic regions,² possibly becoming linked to the basic bone substance through the action of the mucopolysaccharides at the time calcium is laid down.

Independently of our own investigations, others,^{3,4} beginning in 1957 and 1958, encountered fluorescence in the bones of animals and in tumors, and also suggested a possible linkage through the mucopolysaccharides or with calcium.



FIG. 4. Bones from a chicken that had received oxytetracycline for six months. Clearly shown is the marked yellow fluorescence throughout the bone, which contrast with the normal blue of the cartilage.

In the present paper we do not take up the manner in which oxytetracycline becomes linked to bone tissue, since this question is still under study, but we do report several points that suggest the possibility of utilizing oxytetracycline as a means of protecting bone against infection.

The importance of the fact that fluorescent oxytetracycline is deposited and stored for long periods of time in osteogenetic regions, particularly in those of children and youth in whom osteomyelitis is most frequently encountered, can be readily appreciated.

Even though up to now oxytetracycline has been employed extensively in the treatment of various osseous infections, including osteomyelitis caused by *Staphylococcus aureus* and others, the principal aim has always been a suitable concentration of the drug in the blood. In the course of our studies we encountered no suggestion that it might be possible to protect those areas of the bone which take up the antibiotic, where it is incorporated into the osseous trabeculae themselves, either through the action of the mucopolysaccharides directly or through interaction with calcium or with other ions.

Our findings in osteomyelitis indicate that the antibiotic is localized and stored in a highly selective fashion in the osteogenetic regions, which are quite numerous in osteomyelitis, and suggest the possibility of a therapeutic approach which might be termed "protecting the areas of fluorescent bone tissue."

Since we also found that specimens of spongy bone taken from the iliac crest to be used as grafts, as well as those from curetted osteolytic areas in osteomyelitis show marked yellow fluorescence (induced by the deposition of oxytetracycline previously received by the patient), we suggest that implants of this type might be termed "fluorescent iliac grafts," and that they may be presumed to be protected from osteomyelitis by virtue of the antibiotic that is deposited in their trabecular structure.

In view of this selective concentration of fluorescent oxytetracycline in both normal and pathological (as in osteomyelitis) osteogenetic regions, we believe that the practical possibility of protecting the fluorescent areas that take up the antibiotic merits consideration. It may be pointed out that this selective localization theoretically should produce rapid and total recovery, since the antibiotic is concentrated in the very focus of infection, but that actually such a result is far from certain in practice. This fact, however, may be due to the fact that the pathogen has acquired sufficient resistance to affect the therapeutic outcome; or, as we have previously suggested,² it may possibly be due to the fact that the antibiotic is deposited in a non-bacteriostatic form.

We believe, however, that the possibility of protecting fluorescent regions in osteomyelitis may have practical merit; even in the event that the antibiotic becomes neutralized in deposition, for with the process of decalcification (i.e., osteoporosis, osteolysis) in the advanced stages of infection the fluorescent oxytetracycline may be liberated with the loss of calcium and oxytetracycline may at that time regain its bacteriostatic potential in situ. This possibility suggests that it may have prophylactic potential in children and youth, particularly since the antibiotic remains in the bones for months.

This same approach might be useful in the case of portions of the iliac crest used as grafts; besides filling the graft site with plastic material, the implant might liberate the antibiotic in situ as the trabeculae in which it is stored are resorbed by the granulation tissue.

It should be noted that, since oxytetracycline is deposited selectively and retained in osteogenetic regions for considerable periods of time, in practice the drug might be administered in much smaller dosages (than those customarily employed to attain significant blood levels) for short periods, followed by periods of some months when the drug is not administered.

We administered one 250 mg. capsule daily, rather than the usual dosage of 1 Gm. daily. This dosage was given daily for one week, then every other day for the subsequent two weeks.

This dosage cycle could then be repeated, then followed by periods of non-administration of up to three or four months.

Since oxytetracycline appears to be metabolized in the same manner as calcium, we believe it might be possible to enhance its deposition in the bones by administering it in conjunction with vitamin D, but in lower dosage and for more prolonged periods, which could include those during which we suspended administration of the antibiotic. However, at this time we have no evidence on the merits of this approach.

The appearance of fluorescence similar to that of oxytetracycline in the epithelial margins of the fistulas of patients involved in this study and its absence from granulating areas of fistulas, seems to us to be of some interest. We observed also that a similar fluorescence appears in areas moistened by secretions, as well as on the plaster casts of patients having fistulas or skin ulcerations who are under oxytetracycline therapy (fig. 5). Dressings used on such lesions show the same type of fluorescence, which tends to localize on the periphery of the moistened area. This phenomenon may indicate that the antibiotic is eliminated in the secretions, then absorbed by the gauze or plaster casts. This question, however, remains to be clarified.

SUMMARY

1. The yellow fluorescence of oxytetracycline in osteogenetic regions of osteomyelitis is described. The possibility of employing the antibiotic, which is selectively deposited and stored in such areas, as a prophylactic measure is suggested.

2. The possibility of protecting grafts taken from the iliac crest against osteomyelitic infection is also suggested.

3. A therapeutic approach based upon the preferential deposition and storage of oxytetracycline in the bones, employing greatly reduced dosages of the antibiotic for short periods, followed by some months of non-administration of the drug, and the possibility of combining the antibiotic with vitamin D, on the basis that the vitamin might have the same effect upon this drug as it does upon calcium, is outlined. The underlying therapeutic rationale of this approach is the concentration of fluorescent oxytetracycline in osseous tissue.

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The Excretion of Kanamycin in Bile and Pancreatic Fluid

FREDERICK W. PRESTON, MILTON SILVERMAN,
GEORGE C. HENEGAR, AND EDWARD NEVERIL

*Departments of Surgery and Bacteriology, Veterans Administration Research
Hospital, and Northwestern University Medical School, Chicago, Ill.*

Kanamycin is a water-soluble antibiotic active against mycobacteria and gram-positive and gram-negative organisms. It was discovered by Umezawa and associates⁴ in 1957. The present study was undertaken to provide data on concentrations in bile and pancreatic fluid after intramuscular injection of the drug.

METHODS

Kanamycin excretion was studied in 4 patients with T-tube drainage of bile from the common bile duct and in 1 patient with T-tube drainage of pancreatic fluid from a pancreatic fistula.

Kanamycin sulfate was given as a single intramuscular injection of 1 Gm. in 3 ml. of an aqueous suspension. The concentration of kanamycin in serum and bile or in pancreatic fluid was determined before and at 1 or 2 hour intervals after the injection for 12 to 13 hours. Blood samples obtained by venipuncture were allowed to clot and the serum was drawn off, using sterile precautions. Bile and pancreatic fluid samples were sterilized by immersing a sterile plugged tube containing a 3 ml. sample in boiling water for three minutes. A precipitate, if any, was centrifuged and the supernatant used for testing purposes.

Assays were performed by a doubling tube dilution method using a nutrient broth buffered at pH 8 with Tris buffer. Bile was added to the medium to make a 5 per cent solution for assays of bile. The standard test microorganisms recommended for kanamycin assay were inhibited by bile and could not be used for bile assays. Microorganisms isolated from the clinical laboratory were selected on the basis of their ability to grow in buffered nutrient broth containing 5 per cent bile. These were further tested for sensitivity to kanamycin. Organisms sensitive to 1 μ g. or less were selected for use in the assay.

A complete assay consisted of a titration to determine the sensitivity of the test organism using known concentrations of kanamycin. A titration of each test sample of bile, pancreatic fluid, or serum using this microorganism was then done to determine the amount of kanamycin in the sample.

CASE REPORTS

Case 1. A 66 year old man was operated on for stones in the gall bladder. The liver appeared normal at laparotomy, and hepatic function was within normal limits. Cholecystectomy and choledochostomy with T-tube drainage of the common duct were done. The kanamycin excretion study was done on the twelfth postoperative day.

TABLE I

*Kanamycin in Serum and Bile in a Patient after Removal of Gall Bladder with Stones
(Liver Function Normal) (Case 1)*

Hours after intramuscular injection, 1 Gm.	Serum, μg./ml.	Bile		
		μg./ml.	Volume, ml.	Cumulative total μg.
1	20			
2	5	<2.5	25	
3	2.5			
4	0.6	10	30	300
6	0.2	20	10	500
8		10	60	1100
10		5	60	1400
12		2.5	75	1590

Case 2. A 63 year old man was operated on for obstructive jaundice. Cholecystectomy and choledocholithotomy were done. A T-tube was placed in the common bile duct. The liver appeared normal at operation, and hepatic function tests were within normal limits. The kanamycin excretion study was done on the twenty-third postoperative day.

Case 3. A 40 year old man was operated on for chronic pancreatitis. The liver and biliary system appeared normal at operation. Cholecystectomy was done, and a T-tube was placed in the common bile duct. The kanamycin excretion study was done on the ninth postoperative day.

Case 4. A 56 year old man was operated on for a stricture of the common bile duct, cholangitis, and biliary cirrhosis. A previously made choledochoduodenostomy was found intact. T-tube drainage of the common bile duct was instituted. The liver had the gross appearance of biliary cirrhosis. The kanamycin excretion study was done on the fifteenth postoperative day. At this time, liver function tests indicated impairment of hepatic function. They were as follows: Bromsulfalein retention 45 minutes after injection of 5 mg./Kg. was 30.5 per cent; total serum bilirubin was 7.7 mg./100 ml.; alkaline phosphatase was 25 Bodansky units; total serum protein was 7.0 Gm./100 ml.; and serum albumin was 3.3 Gm./100 ml.; blood prothrombin content was 100 per cent of normal. Liver biopsy showed chronic inflammation and biliary cirrhosis.

Case 5. A 44 year old man was operated on for chronic pancreatitis and a pancreatic pseudocyst. A pancreatic duct opening into the cyst was identified, and drainage of pancreatic

TABLE II

*Kanamycin in Serum and Bile in a Patient after Removal of Gall Bladder with Stones
(Liver Function Normal) (Case 2)*

Hours after intramuscular injection, 1 Gm.	Serum, μg./ml.	Bile		
		μg./ml.	Volume, ml.	Cumulative total μg.
1	20			
2	5	1.3	45	59
3	2.5			
4	1.25	5.2	55	345
5	1.25			
6	<1.25	20.5	60	1575
8		41.0	35	3010
10		20.5	60	4240
12		20.5	30	4855

TABLE III

*Kanamycin in Serum and Bile in a Patient with Pancreatitis
(Liver Function Normal) (Case 3)*

Hours after intramuscular injection, 1 Gm.	Serum, μg./ml.	Bile		
		μg./ml.	Volume, ml.	Cumulative total μg.
2	6.2	6.2	25	155
3	3.1	12.5	25	468
4	3.1	12.5	30	843
6	<3	12.5	60	1593
7	<3			
8	<3	6.25	60	1968
10	<3	<6	120	
12	<3	<6	75	

juice from the duct was observed. The cyst was anastomosed to a divided loop of jejunum. A T-tube was placed in the cyst, and the long arm brought to the outside via a jejunostomy through a stab wound in the abdominal wall. Pancreatic fluid drainage through the T-tube varied from 50 to 200 ml. daily. The kanamycin excretion study was done on the eighteenth postoperative day.

RESULTS

Maximum serum levels were first observed one or two hours after intramuscular injection. They ranged from 6.2 to 24.0 μg./ml.

Kanamycin in Bile. Detectable amounts of kanamycin were observed as early as two hours after injection. Maximum concentrations were as follows: Case 1: six hours after injection; case 2: eight hours; case 3: three, four, and six hours; and case 4: three, five, and seven hours. The maximum levels of kanamycin in bile were equal to one half the maximum serum levels in the patient with impaired liver function (case 4). They were the same as the maximum serum levels in case 1, and were twice the maximum serum levels in cases 2 and 3. In 3 of the 4 patients, kanamycin was present in bile 12 or 13 hours after injection. Total amounts of kanamycin recovered in bile were less than 0.5 per cent of the total dosage given. The results are given in tables I to IV.

TABLE IV

Kanamycin in Serum and Bile in a Patient with Impaired Liver Function (Case 4)

Hours after intramuscular injection, 1 Gm.	Serum, μg./ml.	Bile		
		μg./ml.	Volume, ml.	Cumulative total μg.
1	12	<6	2	<12
2	24			
3	24	12.5	20	250
4	24			
5	12	12.5	10	375
7	12	12.5	15	563
9	6	6.25	15	656
11	3	6.25	10	719
13	<3	6.25	15	812

TABLE V

Kanamycin in Serum and Pancreatic Fluid in a Patient with Chronic Pancreatitis (Case 5)

Hours after intramuscular injection, 1 Gm.	Serum, $\mu\text{g.}/\text{ml.}$	Pancreatic fluid		
		$\mu\text{g.}/\text{ml.}$	Volume, ml.	Cumulative total $\mu\text{g.}$
1/2	19			
1	19			
2	5	0.16	5	1
3	2			
4	1	2.5	2	6
6	0.3	5.0	5	31
8		5.0	5	56
10		2.5	10	81
12		2.5	10	106

Kanamycin in Pancreatic Fluid. The antibiotic was detected in pancreatic fluid two hours after intramuscular injection. The maximum concentration, equaling approximately 25 per cent of the maximum serum concentration, was observed six and eight hours after injection. Approximately 0.01 per cent of the total amount injected was recovered in pancreatic fluid. These results are given in table V.

DISCUSSION

Hewitt and Finegold² have previously observed the excretion of kanamycin in human bile, and Tisch and co-workers³ found measurable amounts of the drug in canine hepatic bile. Allowing for the loss of some bile directly into the duodenum by escape around the T-tube, our studies indicate that less than 1 per cent of a 1 Gm. intramuscular dose of kanamycin was eliminated in hepatic bile.

Welch and associates⁵ showed that 51.7 per cent of a 1.0 Gm. intramuscular dose of kanamycin was recovered in urine during the first 24 hours after medication. Cronk and Naumann¹ found that approximately 80 per cent of intramuscular doses of 0.25, 0.5, and 1.0 Gm. were recovered in the urine in this interval. It is therefore apparent that the kidney is chiefly responsible for the excretion of kanamycin and that the liver is not important in this regard.

In 3 patients with normal liver function, the highest concentration of kanamycin in bile was equal to or greater than the highest concentration in the serum. These concentrations of the antibiotic are bactericidal to most strains of *Staphylococcus aureus*, *Aerobacter aerogenes*, and *Escherichia coli* and are inhibitory to strains of *Proteus*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis*.^{5,6} There is no evidence that inactivation or binding of kanamycin by bile occurs. Kanamycin is active at pH 7.5, which is the pH of hepatic bile. These considerations indicate that kanamycin is a suitable antibiotic for use in patients with normal liver function for the control of infections of the biliary tract. The amount of kanamycin in bile in 1 patient with impaired liver function was less than the serum level. However, the level in the bile of this patient was sufficiently high to be bactericidal to all strains of *Staph. aureus* tested by Welch and co-workers⁵ and were within the range given for bacteriostasis for some strains of *A. aerogenes* and *Proteus vulgaris*. It is therefore ap-

parent that the concentration of the antibiotic in bile is related to hepatic function and that in patients with impaired liver function, the drug can be expected to be less effective than in patients with normal function.

Although the peak concentration of kanamycin in pancreatic fluid was only 25 per cent of the peak concentration in serum, the amount was within the range found to be bacteriostatic or bactericidal to most strains of *Staph. aureus* tested by Welch et al⁵ and within the bacteriostatic range for some strains of *A. aerogenes* and *P. vulgaris* tested by Welch et al⁵ and for *Proteus* species and *E. coli* tested by Yow and Monzon.⁶ Kanamycin therefore would appear to be an effective drug for the elimination of bacteria from pancreatic fistulas infected with organisms sensitive to this antibiotic.

SUMMARY AND CONCLUSIONS

Kanamycin was detected in bile as early as two hours after the intramuscular injection of 1 Gm. The level in bile rose rapidly. Maximum levels, one half to two times maximum serum levels, were obtained three to eight hours after injection. Detectable amounts of kanamycin were observed as late as 13 hours after injection.

Kanamycin was recovered in pancreatic fluid two hours and as late as 12 hours after intramuscular injection. Maximum concentrations, amounting to 25 per cent of the maximum serum levels, were observed six and eight hours after injection.

ACKNOWLEDGMENT

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Like streptomycin, dihydrostreptomycin, viomycin, and neomycin, kanamycin can be classified as belonging to the group of basic *Streptomyces* antibiotics.

Continuing our investigations into the pharmacology of these antibiotics, we have studied the toxicological properties of kanamycin in detail and tested ways to reduce these side effects¹⁻⁷ by pantothenic acid. In these experiments kanamycin mono-, di-, and tripantothenate were used. The two former compounds were tested in the basic (pH 7.8 to 8.0) as well as the neutral form (pH 6.8 to 7.0), the difference of the salts being half a SO_4 group added in the neutral form. All these compounds and the corresponding kanamycin sulfates were prepared from the same basic material and contained the same amount of kanamycin A and B (A 98 per cent, B 2 per cent).

In table I are shown the LD_{50} values of the basic kanamycin compounds in comparison with kanamycin sulfate after intravenous, subcutaneous, and oral administration in white mice, the data having been analyzed according to the method of Litchfield and Wilcoxon.⁸ It may be seen that the pantothenates are significantly less toxic than the sulfate form in intravenous dosage. Subcutaneously, the differences are less marked. The oral LD_{50} could not be determined owing to technical reasons, the required amounts being so large that administration even in the form of a suspension would not be practicable.

In the acute trial the LD_{50} values of neutral kanamycin compounds (pH 6.8) are less favorable compared with those of the basic compounds (pH 8.2). The LD_{50} values after intravenous administration in white mice averaged 185 mg. base per Kg.; after subcutaneous administration they averaged 1420 mg. base per Kg., this experiment showing no significant difference between kanamycin pantothenates and sulfate. The less favorable acute toxicity of the neutral compounds was proved to be caused by the influence of the pH . Shifting the pH from 8.2 to 6.8 increases the acute toxicity in all four kanamycin compounds. Calcium also influences the acute toxicity of kanamycin: Addition of more than 0.5 per cent, referred to kanamycin base, lowers the toxicity. This influence of the pH and the effect of calcium are independent of animal species and mode of administration.

To assess the semichronic toxicity of kanamycin pantothenate in comparison with that of kanamycin sulfate, white male mice were treated subcutaneously with daily dosages of 400 mg. base per Kg. of the neutral kanamycin compounds. As is shown in table II, after 45 days 30 per cent of the animals treated with kanamycin sulfate had died, whereas in the same period the group treated with kanamycin monopantothenate had 20 per cent deaths, the dipantothenate group no deaths, and the tripantothenate group 5 per cent deaths.

These data demonstrate, in the semichronic study, a significant superiority of the pantothenates, especially the di- and tripantothénées, as compared with the sulfate.

TABLE I

Acute Toxicity of Kanamycin Sulfate and Pantothenates in Mice

	LD ₅₀ mg. base per Kg. mouse		
	Intravenous	Subcutaneous	Oral
Kanamycin monosulfate	225 (217-233)	1850 (1740-1965)	> 10,000
Kanamycin monopantothenate	245 (233-258)	1950 (1815-2095)	> 10,000
Kanamycin dipantothenate	265 (252-279)	2000 (1845-2165)	> 10,000
Kanamycin tripantothenate	245 (233-258)	1850 (1740-1970)	> 10,000

pH 8.2.

Calcium <0.49 per cent per base.

 $p = 0.05$.

The question of the effect of chronic administration of kanamycin pantothenate and sulfate on the vestibular function was studied in various animal species by testing postrotatory nystagmus and using the swimming test of Caussé, Gondet, and Vallancien modified by Brigham and Nielsen.⁹ In figure 1 are presented the results of an experiment with white mice that clearly shows the effect of kanamycin. It is evident from this experiment that doses of 400 mg. base per Kg. of basic kanamycin pantothenates and sulfate given subcutaneously five times/week did not cause significant damage in the vestibular function of white mice, whereas equal doses of streptomycin sulfate produced severe vestibular injury, as was demonstrated by the complete loss of postrotatory nystagmus and of swimming ability. Similar experiments in rats and guinea pigs, extended over 67 and 61 days respectively, have confirmed that kanamycin under such experimental conditions does not substantially affect vestibular function.

The specific chronic toxicity of kanamycin pantothenate for the cochlear nerve was examined in rats using the Preyer ear postural reflex test and the conditioned reflex test of Courvoisier and Leau.¹⁰

The experimental results using the Preyer reflex test can be seen in figure 2: The hearing loss, expressed in decibels, of the animals treated with basic kanamycin compounds for 17 weeks is shown in experiment A. The dosage was 400 mg. base per Kg. in two equal doses given daily five times/week. In experiment B the neutral compounds were tested using a dosage of 2×150 mg. base per Kg. five times per

TABLE II

Semichronic Toxicity of Kanamycin Sulfate and Pantothenates in Mice

	Dose, mg. base per Kg. subcutaneous	No. mice	Mortality, per cent, on day						
			15	20	25	30	35	40	45
Kanamycin 1½ sulfate	400	20	0	20	20	20	20	25	30
Kanamycin monopantothenate monosulfate	400	20	0	10	15	20	20	20	20
Kanamycin dipantothenate semisulfate	400	20	0	0	0	0	0	0	0
Kanamycin tripantothenate	400	20	0	0	5	5	5	5	5

pH 6.8.

Calcium <0.26 per cent per base.

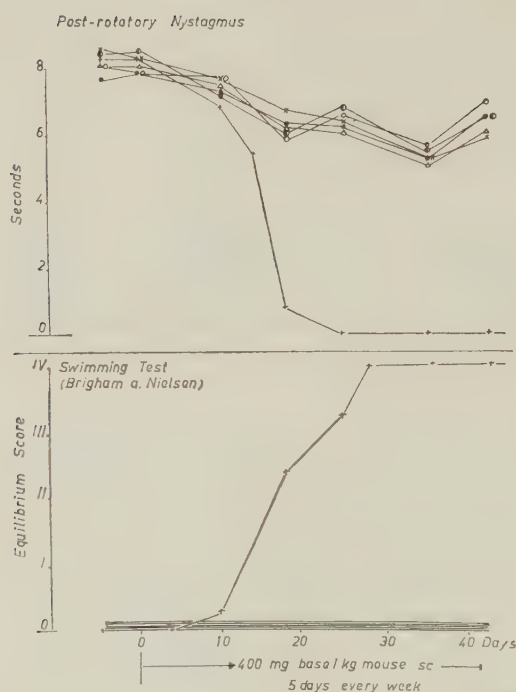


FIG. 1 The effect of kanamycin sulfate (Δ — Δ), kanamycin monopantothenate (\circ — \circ), kanamycin dipantothenate (\bullet — \bullet), kanamycin tripantothenate (\bullet — \bullet), and streptomycin sulfate ($+$ — $+$) on the vestibular function of mice. Controls, \times — \times .

week for 11 weeks. As is shown, kanamycin sulfate caused severe damage to auditory function in both experiments, whereas there was significantly less auditory loss in animals treated with kanamycin dipantothenate. Less favorable were kanamycin mono- and tripantothenate, which caused cochlear damage as severe as that after treatment with kanamycin sulfate. In the same experiment equal doses of dihydrostreptomycin sulfate showed almost no toxic influence on the Preyer reflex.

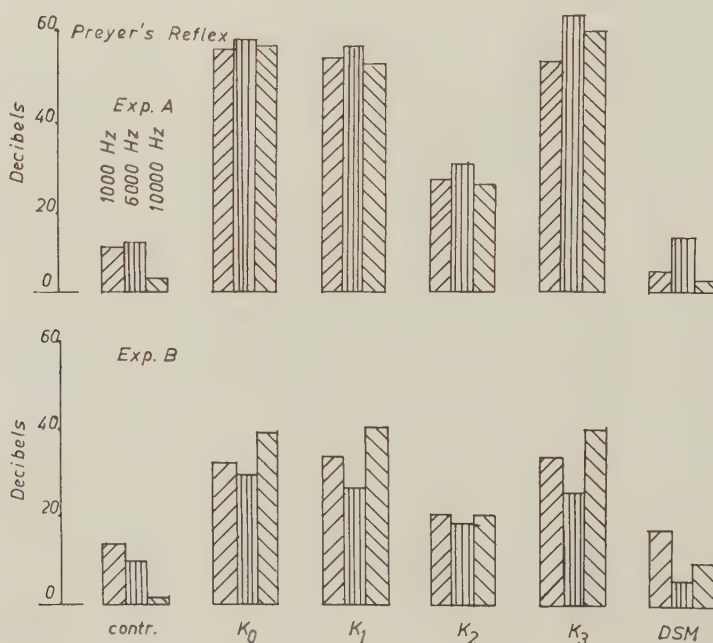
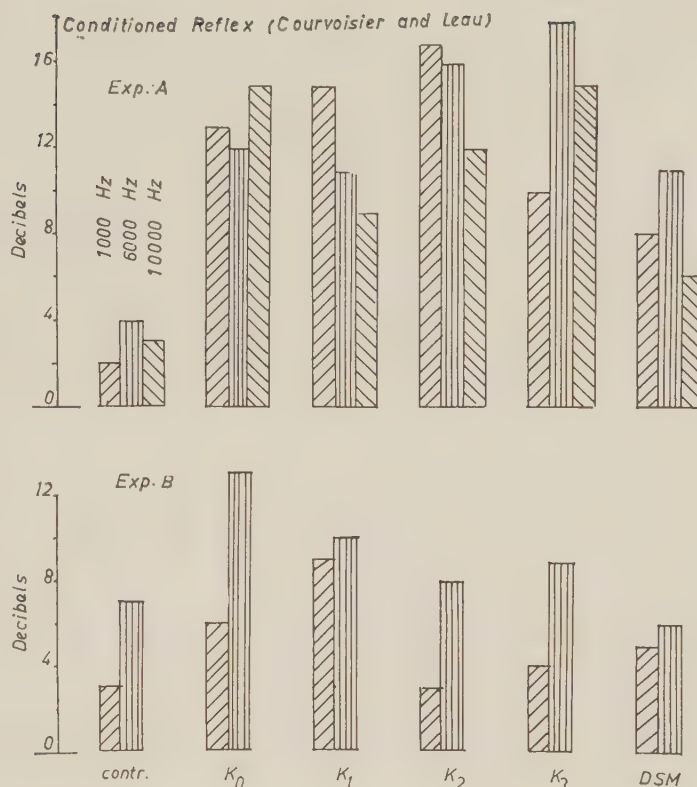


FIG. 2. Effect of kanamycin sulfate (K_0), kanamycin monopantothenate (K_1), kanamycin dipantothenate (K_2), kanamycin tripantothenate (K_3), and dihydrostreptomycin sulfate (DSM) on the auditory function of rats. Experiment A (top): Loss of hearing in rats after 17 weeks' treatment with 2×200 mg. base/Kg./day, except Saturday and Sunday. Experiment B (bottom): Loss of hearing in rats after 11 weeks' treatment with 2×150 mg. base/Kg./day subcutaneously.

FIG. 3. Effect of kanamycin sulfate (K_0), kanamycin monopantothenate (K_1), kanamycin dipantothenate (K_2), kanamycin tripantothenate (K_3), and dihydrostreptomycin sulfate (DSM) on the auditory function of rats. Experiment A (top): Loss of hearing in rats after 17 weeks' treatment with 2×200 mg. base/Kg./day, except Saturday and Sunday. Experiment B (bottom): Loss of hearing in rats after 11 weeks' treatment with 2×150 mg. base/Kg./day subcutaneously.



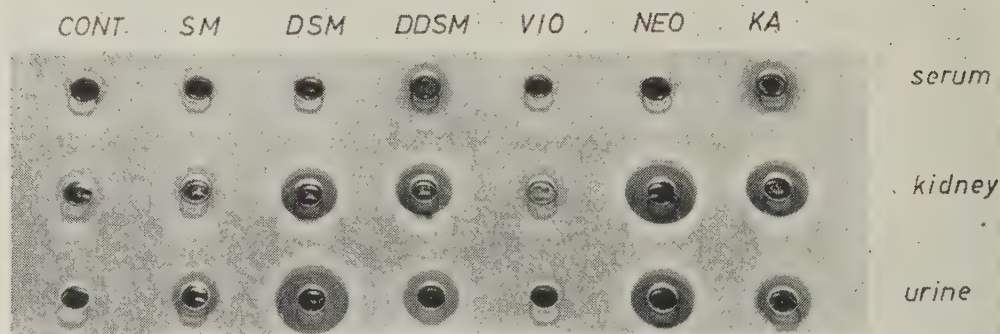
The Courvoisier test (figure 3) essentially confirmed the toxicity-reducing effect of kanamycin dipantothenate in experiment B, but not in experiment A, which fact may be partly explained by dosage and duration of the experiment. Furthermore, bearing in mind that the Courvoisier test employs a complex reaction system whereas the Preyer reflex is a far simpler auditory reflex, it might be conceded that certain differences in the results are to be expected.

The ototoxicity of kanamycin pantothenate compared with that of kanamycin sulfate was also tested in guinea pigs. The auditory function of these animals was assessed by Preyer reflex test and by microphonic reaction. The results, identical by both methods, showed that subcutaneous treatment with 100 mg. kanamycin monopantothenate per Kg. per day for six weeks, followed by an interval of three weeks without treatment, did not cause auditory loss whereas kanamycin sulfate produced marked damage to hearing ability. In guinea pigs kanamycin di- and tripantothenate proved to be less favorable than kanamycin monopantothenate.* A comment on a potential late damage cannot be given at present.

On the basis of the results obtained in rats and guinea pigs using the Preyer test and the microphonic reaction, we conclude that pantothenic acid significantly reduces the cochleotoxic side effects of kanamycin in animal experiments. The detoxifying effect of pantothenic acid is dependent on its concentration, and the opti-

* These experiments have been carried out in collaboration with Dr. Tyberghein, University Clinic "St. Raphael," Louvain, Belgium.

after 24 hours



after 48 hours

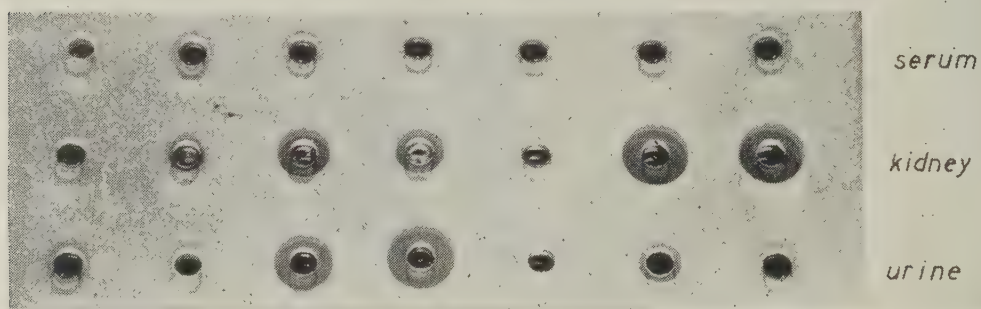


Fig. 4. Serum, kidney, and urine levels of streptomycin (SM), dihydrostreptomycin (DSM), dihydrodesoxystreptomycin (DDSM), viomycin (VIO), neomycin (NEO), and kanamycin (KA) in rats 24 and 48 hours after a single subcutaneous injection of 20 mg. base/Kg.

imum amount of pantothenic acid varies with the susceptibility of the animal species tested.

Investigations of the specific chronic toxicity to the kidney in rats showed that kanamycin—like neomycin—is accumulated to a certain degree in the kidneys and is excreted only very gradually. This was shown in a microbiological activity assay (fig. 4) of kanamycin sulfate in serum, in urine, and in the kidney after a single subcutaneous dose of 20 mg. base per Kg. In addition to kanamycin, neomycin, streptomycin, dihydrostreptomycin, dihydrodesoxystreptomycin, and viomycin were tested, the results obtained with the latter antibiotic being not conclusive, since the sensitivity to viomycin of the assay strain *Bacillus subtilis* ATCC 6633 proved to be too small. Twenty-four hours after administration of kanamycin sulfate no further activity was demonstrable in the serum. After 48 hours the urine levels also had fallen below measurable amounts, whereas in the kidney, levels of 10 $\mu\text{g./Gm.}$ could be demonstrated even after 72 hours, neomycin showing similar results. Streptomycin could not be detected in either the serum, the kidney, or the urine after 24 hours; dihydro- and dihydrodesoxystreptomycin had only a somewhat delayed excretion.

The chronic administration of kanamycin sulfate caused renal damage in rats as demonstrated by albuminuria and a deposit of leukocytes, renal epithelia, and

granulated and hyaline casts. The damage was reversible to a great extent. So far there seems to be no effect of pantothenic acid on the nephrotoxicity of kanamycin.

SUMMARY

In conclusion, we feel that our experiments show that pantothenic acid is capable of reducing the acute, semichronic, and cochlear toxicity of kanamycin, under the conditions described.

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Comparative Clinical Evaluation of Sulfaphenazole, Sulfadimethoxine, and Sulfamethoxypyridazine

A. RUNE FRISK AND ÅKE HOLMGÅRD

*Medical Service, Ersta Hospital, and Central Laboratory,
St. Eriks' Hospital, Stockholm, Sweden*

Chemotherapy with sulfonamide preparations has gained a renewed interest in the last few years, especially through the discovery of compounds that possess pharmacological properties essentially superior to those used earlier. The first of these was sulfamethoxypyridazine,* the pharmacological properties of which we have described in earlier papers.^{1,2} After the introduction of this drug, a number of other sulfonamide derivatives with similar chemotherapeutic properties were studied. Two of them, sulfaphenazole^{3†} and sulfadimethoxine,^{4,5‡} have proved promising. A comparative study of the absorption and excretion of these three sulfonamide derivatives after single and repeated doses in man is presented in this paper.

METHOD AND MATERIAL

A single dose of 4 Gm. of these sulfonamide derivatives was given on three separate occasions to each of 3 adult convalescent patients with normal renal function and without signs of cardiac or gastrointestinal disorder. The drugs were given on an empty stomach, after which the concentrations in blood and urine were recorded at specific intervals. In a similar group of patients the blood concentration was studied after repeated administration of 1 Gm. sulfaphenazole and sulfadimethoxine every twelfth hour. Blood tests were taken in these cases immediately before the morning dose and therefore represent the lowest concentration during the 24 hours. The results are compared with those we reported previously for sulfamethoxypyridazine under the same test conditions.¹ Finally, in a third group of patients, the renal clearance was determined concurrently with creatinine clearance and, in addition, the degree of protein-binding of the different substances. The same methods as we used earlier were used for determination of the action of the sulfonamide derivatives and for conducting the tests.⁶

RESULTS

Table I shows blood concentration and urinary excretion after a single 4 Gm. dose of the various drugs to each of 3 experimental persons. The values are means, and the individual results are in close agreement. All three substances were quickly absorbed, sulfaphenazole perhaps most quickly, its maximum concentration in the blood coming after about four hours. With sulfadimethoxine and sulfamethoxypyrid-

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for sulfamethoxypyridazine is Lederkyn.

† The trade name of Ciba Pharmaceutical Products for sulfaphenazole is Orisulf.

‡ The trade name of Hoffmann-LaRoche for sulfadimethoxine is Madribon.

azine, the highest concentrations came rather later. Sulfamethoxypyridazine gives the greatest and most long-lasting concentration, sulfadimethoxine gives a somewhat lower concentration, and sulfaphenazole the lowest. This is further illustrated in figure 1. The three drugs acetylate in the blood only to a slight degree. Sulfaphenazole perhaps acetylates to a rather greater extent than the other two. Excretion through the kidneys proceeds slowly. Sulfaphenazole is excreted more quickly than sulfadimethoxine and sulfamethoxypyridazine. Roughly one third of the substance recovered from the urine occurs in conjugated form. The various drugs are all fairly alike in this respect.

The concentration in urine very seldom exceeds 150 mg./100 ml., even under conditions of low diuresis, so that the risk of precipitation of sulfonamide crystals from these drugs in tubules is virtually negligible.

The blood concentration under continuous treatment with sulfaphenazole was studied in 15 cases. The results were compared with those obtained in 15 other patients to whom sulfadimethoxine was given on a similar dosage schedule and with the results reported by us for sulfamethoxypyridazine in the same dosage (table II). The dosage was 1 Gm. every twelfth hour. The values represent the lowest concentration during the 24 hours. As seen from table II, sulfaphenazole shows a far lower

TABLE I

Absorption and Excretion of Sulfaphenazole, Sulfadimethoxine, and Sulfamethoxypyridazine after Single Oral Doses in the Same 3 Persons (Mean Values)

Dose, Gm.	Time,* hours	Blood		Urine				
		Concentration, mg./100 ml.		Concentration, mg./100 ml.		Excretion of drug, mg./period		Per cent of administered dose excreted, cumulative total
		Free	Total	Free	Total	Free	Total	
4, sulfa- phenazole	2	4.8	5.9	14	20	24	39	1
	4	5.3	6.5	101	140	221	315	8
	8	5.0	5.5	149	164	666	904	22
	12	2.4	2.9	158	178	1,034	1,430	36
	24	1.8	2.6	119	179	1,547	2,155	56
	48	1.0	1.5	42	68	2,017	2,887	72
	72	0.6	0.9	23	38	2,215	3,207	80
	96	—	—	13	32	2,352	3,443	85
4, sulfa- dimeth- oxine	2	4.5	4.9	5	6	17	25	0.5
	4	8.3	9.0	28	37	45	68	1.5
	8	10.7	11.4	38	56	157	229	5
	12	8.9	9.6	48	79	270	423	10
	24	4.9	5.3	68	106	694	1,083	27
	48	2.9	3.2	37	50	1,180	1,746	43
	72	1.5	2.5	27	39	1,567	2,303	58
	96	0.7	2.0	22	34	1,793	2,646	66
4, sulfa- methoxy- pyridazine	2	10.3	11.8	18	32	30	53	1
	4	16.4	17.9	74	126	88	151	3
	8	16.1	17.2	62	107	201	339	8
	12	12.9	13.8	68	120	394	641	16
	24	11.2	11.9	62	127	695	1,282	32
	48	8.4	9.8	35	72	1,151	2,239	56
	72	4.4	5.4	33	59	1,481	2,821	70
	96	3.4	4.1	28	40	1,704	3,188	80

* All times recorded from beginning of study.

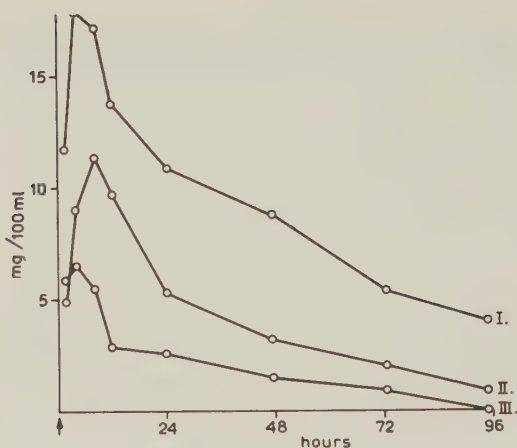


FIG. 1. Blood concentration after single oral dose. I, sulfamethoxypyridazine; II, sulfadimethoxine; III, sulfaphenazole.

mean concentration than sulfadimethoxine and sulfamethoxypyridazine. There is less difference in blood concentration between the two latter compounds, as will be seen also from figure 2.

In all these cases we kept a daily check on the occurrence of sulfonamide crystals in sediment. No such crystallization was found, nor did we record any effect on renal function. As will be seen from table II and figure 2, there is no appreciable risk of accumulation with the dosage employed in this study. Nevertheless, the schedule resulted in unnecessarily high sulfonamide concentrations when sulfadimethoxine and sulfamethoxypyridazine were used, and consequently the risk of toxic side effects was increased. A dosage of 1 to 2 Gm. of the two latter drugs during the first day, thereafter 0.5 to 1 Gm. every morning, should suffice for effective therapy. Sulfamethoxypyridazine could undoubtedly be given in lower dosage than sulfadimethoxine.

Renal clearance of sulfadimethoxine and sulfaphenazole was determined concurrently with creatinine clearance. At the same time the quantity of the non-protein-bound, ultrafiltrable part of the compounds was determined. The experimental patients were convalescents with healthy kidneys. They were given 1 Gm. of the various compounds two hours before the start of the test. Urine was collected quantitatively during two test periods. Ultrafiltrate was prepared by the same method

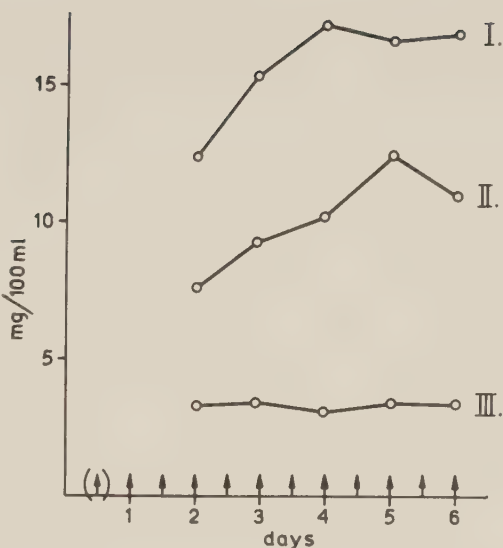
TABLE II

Blood Concentration after Continuous Administration of Sulfaphenazole, Sulfadimethoxine, Sulfamethoxypyridazine (1 Gm. every 12 hours)
(Mean Values, mg./100 ml.)

Drug	Second day		Third day		Fourth day		Fifth day		Sixth day		Number of experiments
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	
Sulfaphenazole	2.9	3.3	2.9	3.4	2.6	3.1	2.8	3.4	2.7	3.4	15
Sulfadimethoxine	7.0	7.7	8.6	9.3	8.6	10.2	11.7	12.5	10.0	11.0	15
Sulfamethoxypyridazine*	11.3	12.4	14.0	15.4	15.2	17.2	14.7	16.7	15.4	16.9	13

* These values have been reported previously.

FIG. 2. Blood concentration after continuous administration of 1 Gm. every 12 hours. I, sulfamethoxypyridazine; II, sulfadimethoxine; III, sulfaphenazole.



as in a previous report.¹ The results are compiled in table III. Clearance of sulfadimethoxine was very low, that of sulfamethoxypyridazine being roughly of the same order; the sulfaphenazole figures are two to three times as high. Owing to the very negligible acetylation of the two compounds, the clearance values for the acetylated drugs were not calculated. Both sulfadimethoxine and sulfaphenazole had high and roughly equal protein-binding, of the same order as sulfamethoxypyridazine.

Sulfaphenazole, sulfadimethoxine, and sulfamethoxypyridazine have similar and beneficial pharmacological properties. They are quickly absorbed and slowly excreted, so that it is easy to establish and maintain an active sulfonamide concentration in the blood with fairly small total dosages. Owing to their slow excretion, the concentrations in urine are seldom such as to entail risk of renal injury. All are acetylated to a slight and roughly equal degree. The plasma protein-binding is high and about equal for all three compounds. Sulfaphenazole is excreted through the kidneys two to three times as quickly as sulfadimethoxine or sulfamethoxypyridazine,

TABLE III
Creatinine Clearance

Drug	Patient no./period	Creatinine clearance, ml./min.	Clearance of free plasma, ml./min.	Ultrafiltrate fraction, per cent
Sulfadimethoxine	I/1	140	3.1	10
	I/2	125	2.6	
	II/1	190	2.1	7
	II/2	171	3.3	
	III/1	144	1.6	10
	III/2	153	1.6	
Sulfaphenazole	IV/1	89	4.7	
	IV/2	105	9.7	6
	V/1	146	9.1	
	V/2	137	10.7	11

and its concentration in the blood is therefore very much lower and of shorter duration than that of the two other drugs. Of the latter, sulfamethoxypyridazine has the greatest and most long-lasting concentration in blood.

ACKNOWLEDGMENT

We wish to thank Lederle Laboratories Division, Ciba Pharmaceutical Products, and Hoffmann-La Roche for placing at our disposal the drugs sulfamethoxypyridazine, sulfaphenazole, and sulfadimethoxine, respectively.

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Effects of Procaine Penicillin in Chickens Monocontaminated with *Clostridium perfringens* and with *Streptococcus faecalis**

BERNARD S. WOSTMANN, MORRIS WAGNER, AND HELMUT A. GORDON

*Lobund Germfree Life Research Laboratories, Department of Biology,
University of Notre Dame, Notre Dame, Ind.*

Young conventional chickens (approximately 1 or 2 months of age), fed antibiotics incorporated in the diet at nutritional levels from birth, display morphological characteristics different from those seen in non-antibiotic-treated conventional birds but qualitatively similar to those seen under germfree conditions.¹ Further study of this effect showed that while the total viable count of intestinal organisms apparently was not reduced by the feeding of nutritional levels of penicillin, the number of *Clostridium perfringens* organisms was markedly reduced and the number of *Streptococcus faecalis* organisms in the lower ileum also showed possible reduction.² No influence on serum gamma globulin was seen in this age group,³ but scattered reticulo-endothelial cells in the wall of the lower ileum showed a substantial reduction.⁴

In view of these bacteriological observations, the effect of dietary penicillin was then studied in chickens monocontaminated with *Cl. perfringens* or with *Str. faecalis*. The presence of *Str. faecalis* as a monocontaminant caused some increase in the total number of scattered reticulo-endothelial elements in the ileum and brought gamma globulin and agglutinin (anti *Str. faecalis*) values up to a level seen in conventional birds. Administration of penicillin had no obvious influence on the number of organisms, serum gamma globulin, agglutinin titers, or scattered reticulo-endothelial elements.^{2,4} Some preliminary data on the effect of penicillin on numbers of organisms in chickens monocontaminated with *Cl. perfringens* have been given in an earlier report.² The studies presented here give additional and more detailed information about the effect of dietary penicillin on chickens monocontaminated with *Cl. perfringens*, particularly from the standpoint of the effect on bacterial population, serum gamma globulin levels, homologous agglutinin titers, and incidence of scattered reticulo-endothelial cells in the ileum wall. Comparisons are made with results from previous observations obtained from *Str. faecalis* monocontaminated and from conventional chickens.²

In addition, a quantitative study of lamina propria tissue was included in this series. A previous comparison of germfree and conventional animals has indicated that the presence of the normal microbial flora imparts certain characteristics to the intestine of the conventional host. Among these, increased weight, hydration,⁵ and richer supply of reticulo-endothelial cells⁴ were noted in various segments of the small intestine. Due to the nature of these characteristics, it was indicated that the presence of the flora maintains a state of "physiological inflammation" in the intestinal wall. In order to broaden this concept, it was decided to study the effect

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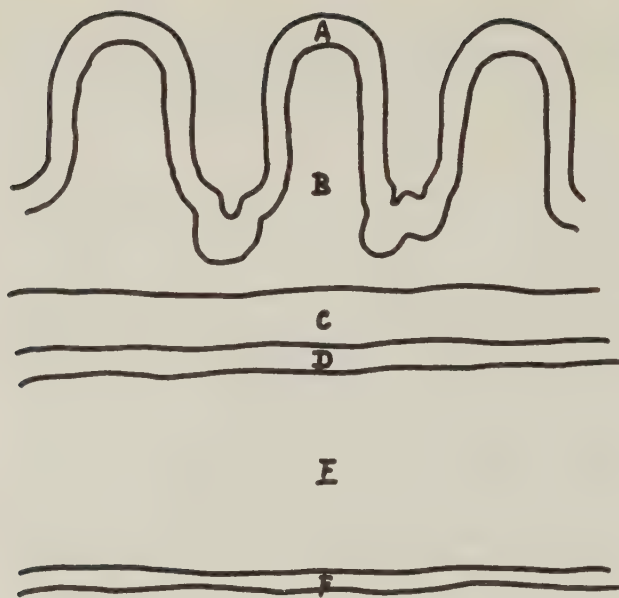


FIG. 1. Diagram of tissue constituents in lower ileum of the chicken. A = epithelium; B = lamina propria; C = muscularis mucosae; D = submucosa; E = muscularis; F = serosa.

of the flora on the connective tissue element of the intestinal wall, which, as is commonly known, is particularly responsive to prolonged bacterial stimulation. Thus, a quantitative study of lamina propria tissue of the intestine was included in this series since this tissue represents the contingent of the gut wall that is richest in connective tissue, and accordingly promised to be a good indicator of the intensity of the bacterial stimulus.

MATERIAL AND METHODS

The experimental animals were, unless stated otherwise, 65 to 70 day old white Leghorn chickens of mixed sexes. The diet, in all experimental groups, was the steam-sterilized, practical-type diet L-289 F, with or without radiation-sterilized procaine penicillin G at 50 mg./Kg. diet.¹ Germfree and monocontaminated birds were housed in Reyniers-type units.⁶ Conventional animals were maintained in the animal room in wire brooders. The monocontaminated animals were obtained

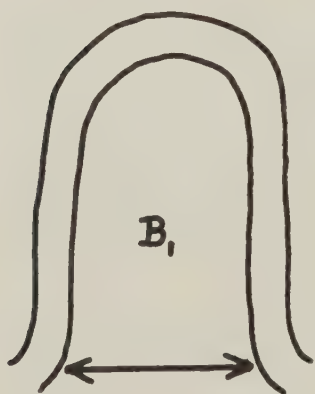


FIG. 2. Diagram of "core of villus" (B₁).

as stated earlier² and divided into two groups within the same germfree unit, one with and one without penicillin in the diet.

Sacrifice was by exsanguination after electronarcosis. Details regarding collection of materials and the procedures for bacteriology,² electrophoresis,⁷ and agglutinin titer determination⁸ have been previously published.

Full details of the quantitative determination of lamina propria tissue will be published elsewhere. In essence, the procedure consists of establishing the lamina propria contingent as a percentage of the total intestinal tissue, using camera lucida and planimeter techniques in histological specimens (cross sections). The material used in this work originated uniformly from a portion of the ileum that is adjacent to the ileocecal colic junction. The Bouin fixed and celloidin-paraffin embedded tissue was cut at 4 μ and stained according to Hansen-van Gieson. The camera lucida drawing included approximately one quarter of the total cross section. The tissues identified as lamina propria in this segment of the chicken intestine are indicated in figure 1. They include, in essence, blood vessels, lacteals, some smooth muscle elements, and mainly supportive tissue. Three individual sections were evaluated in each animal.

"Core of villus," as used in this paper, represents a portion of the lamina propria that extends into the villus. It was subdivided from the rest of the lamina by an imaginary line drawn at the base of the villus, as indicated in figure 2. The value of core of villus is given as a percentage of the total villus area. In each animal, three sections were thus evaluated with four to five villi/section.

The lamina propria and core of villus values ran largely parallel in our observations. Since the latter appears to be a more sensitive indicator of the intensity of the bacterial stimulus, only core of villus values are presented in this paper. The results obtained with the lamina propria will be mentioned in a forthcoming publication.

The technical details of establishing the count of scattered reticulo-endothelial cells in the intestinal wall have been described previously.⁴

RESULTS AND DISCUSSION

Results from previous and present observations have been brought together in table I. An analysis of the various differences found in table I is given in table II. The following conclusions seem to stand out.

In chickens with a mixed "conventional" flora, feeding of 50 mg. procaine penicillin G per Kg. diet results in: no material change in the total viable count of intestinal microorganisms;² a marked reduction in the numbers of *Cl. perfringens* and a possible slight reduction in *Str. faecalis*; an unchanged serum gamma globulin level; a strong reduction of scattered reticulo-endothelial elements in the wall of the lower ileum—this change was limited mainly to lymphocytes, plasma-cells, and Schollen leukocytes; a reduction of lamina propria tissue as indicated by core of villus values (see "Methods") to practically the level seen in germfree birds.

In *Cl. perfringens* monocontaminated birds, the effects of penicillin are the following: a reduction of the number of organisms to only a fraction of that seen in untreated chickens; a lowering of serum gamma globulin levels below the already suboptimal response brought about by *Cl. perfringens* monocontamination—some effect of remaining organisms seems to be reflected by the fact that the level attained

TABLE I

Effect of Dietary Penicillin (PP) on Various Characteristics of Conventional Chickens and of Chickens Monocontaminated with Cl. perfringens and with Str. faecalis (Age 65 Days Unless Indicated Otherwise; Sexes Mixed; Number of Experimental Animals Given Between Parentheses; Standard Deviation of the Mean Values Given)

	Gamma globulin, mg./100 ml.	Organisms in lower ileum, log/Gm. dry content*		Range of agglutinin titers against homologous organisms		Core of villus, % of area†	Total scattered RE elements in ileum wall/sq. mm.‡§
		<i>Cl. perfringens</i>	<i>Str. faecalis</i>	<i>Cl. perfringens</i>	<i>Str. faecalis</i>		
Conventional	800(11) ±55	—	—	—	—	—	—
Conventional plus PP	806 (9) ±85	—	—	—	—	—	—
Conventional	701(12) ±48	5.6 (3) 4.5-6.0	7.6 (3) 6.8-7.9	0-1/2 (6)	1/16 (2)	36.8(17) ±1.4	2860 ±240 (11)
Conventional plus PP	—	2.5 (3) <1.0-2.8	6.4 (3) 6.3-6.5	—	—	27.9 (7) ±1.8	1380 ±130 (6)
<i>Cl. perfringens</i>	534(10) ±33	9.2 (10) 6.9-9.8	—	0-1/4 (10)	—	32.2 (8) ±1.3	2000 ±215 (3)
<i>Cl. perfringens</i> plus PP	416 (7) ±25	2.4 (8) <1.0-2.9	—	0 (8)	—	26.1 (8) ±1.6	1180 ±180 (3)
<i>Str. faecalis</i>	775 (4) ±119	—	10.4 (4) 9.3-10.6	—	1/32-1/128 (5)	30.5 (5) ±0.6	1460 ±150 (6)
<i>Str. faecalis</i> plus PP	764 (4) ±226	—	9.9 (4) 7.5-10.1	—	1/16-1/128 (5)	25.9 (6) ±0.5	1220 ±120 (6)
Germfree	320(12) ±23	—	—	0 (5)	0 (5)	25.6(17) ±0.9	930 ±60 (11)

RE = Reticulo-endothelial.

* Mean and range given. Failure to demonstrate organisms in the most concentrated sample preparations is arbitrarily recorded as <1.0.

† See text.

‡ Combination of about equal numbers of 35 and 65 day old animals.

§ In mucosa and submucosa only.

|| Age 75 days.

is on the average still higher than that seen in germfree birds; a level of agglutinating antibody that for practical purposes was undetectable with the methods used—in 10 monocontaminated animals, titers were 0 (6/10); doubtful titer* (2/10); and 1/4 (2/10); in 8 monocontaminated birds fed penicillin, titers were 0 (7/8) and doubtful (1/8); a neutralization of the strong reticulo-endothelial stimulating effect of *Cl. perfringens*; a reduction of lamina propria tissue from practically “conventional values” to amounts found normally in germfree animals.

In *Str. faecalis* monocontaminated chickens, the following effects were seen: no reduction of the substantial number of microorganisms in the ileum; no change in the high gamma globulin levels evoked by the presence of *Str. faecalis*; no significant change in agglutinating antibody titers; little influence on the mild stimulus of the reticulo-endothelial system by this organism; a reduction of the amount of lamina propria tissue, obviously but “subconventionally” stimulated by the presence of the organism, to germfree levels.

In surveying these results, it would seem that both gamma globulin production and agglutinating antibody titers are largely related to the numbers of organisms present, though our results clearly show *Cl. perfringens* to be less potent than *Str. faecalis* in stimulating a gamma globulin and antibody response.⁹ The same might possibly be true for the total number of scattered reticulo-endothelial elements in the lower ileum. The lamina propria, as indicated by the percentage area of the

* Doubtful titer refers to a trace reaction (incomplete agglutination) that occurred at the lowest serum dilution (1:2) tested.

TABLE II

Analysis of Significance of the Most Important Differences in Table I

	Gamma globulin	Core of villus	Scattered reticulo-endothelial elements in ileum wall/sq. mm.
Conventional vs. <i>Cl. perfringens</i>	s	0.10	0.12
Conventional vs. <i>Str. faecalis</i>	ns	s	s
Conventional vs. germfree	s	s	s
Conventional vs. conventional plus PP	ns	s	s
<i>Cl. perfringens</i> vs. <i>Cl. perfringens</i> plus PP	s	s	s
<i>Str. faecalis</i> vs. <i>Str. faecalis</i> plus PP	ns	s	ns
Conventional plus PP vs. germfree	s	ns	s
<i>Cl. perfringens</i> vs. germfree	s	s	s
<i>Cl. perfringens</i> plus PP vs. germfree	s	ns	ns
<i>Str. faecalis</i> vs. germfree	s	s	s
<i>Str. faecalis</i> plus PP vs. germfree	s	ns	0.18

s means $P \leq 0.01$; s means P value between 0.01 and 0.05; ns means not significant; P values between 0.05 and 0.20 given.

core of villus, seems to offer another picture. In all cases (conventional, *Cl. perfringens*, and *Str. faecalis* monocontaminated) the addition of 50 mg./Kg. procaine penicillin G to the diet brought the amount of tissue back to practically germfree levels. In the case of *Cl. perfringens*, the antibiotic resulted in a strong reduction in numbers of organisms. In the case of *Str. faecalis*, hardly any reduction in number occurred, but the effect of the antibiotic on the lamina propria was still apparent. This would indicate that, especially in the latter case, not numbers of organisms but some change in the organism or its metabolism was the determining factor. Similar conclusions have been drawn by Lev and Forbes¹⁰ from their work on growth depression caused by *Cl. perfringens* in very young chickens. Their data showed that in certain experiments the reduction in the number of organisms seemed to be the determining factor in alleviating growth depression, while in others, where no reduction in number was seen, a changed metabolism of the microorganisms was believed to be responsible for the elimination of the growth depressive effect.

SUMMARY

Germfree hatched chickens monocontaminated directly after birth with *Cl. perfringens*, when compared with germfree chicks, displayed a well-established intestinal population of this organism and a substantial increase in both the percentage of lamina propria tissue and the number of reticulo-endothelial elements in the wall of the lower ileum. Addition of procaine penicillin G at 50 mg./Kg. of diet, when fed to similarly monocontaminated chicks, virtually eliminated the organism from the intestine and maintained the lamina propria tissue and scattered reticulo-endothelial elements at essentially germfree levels. However, a slightly

but significantly higher level of gamma globulin still persisted in the serum of the antibiotic-treated group.

In similar studies with chickens monocontaminated with *Str. faecalis*, no influence of the antibiotic on the number of organisms was seen. Serum gamma globulin and anti *Str. faecalis* agglutinins in both groups (with and without penicillin) were at a level seen in conventional animals. The number of scattered reticulo-endothelial elements was stimulated only mildly by the organism and no influence of the antibiotic was discernible. The amount of lamina propria tissue, however, was strongly increased by the presence of *Str. faecalis* but remained on a germfree level when penicillin was added to the diet.

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The Effect of Calcium Salts on Chlortetracycline Absorption

E. L. R. STOKSTAD, J. M. PENSACK, AND C. N. HUHTANEN

*Research Laboratories, Agricultural Division, American Cyanamid Co.,
Pearl River, N. Y.*

Eisner et al¹ have shown that feeding any of a number of acidic compounds, such as citric acid, mucic acid, and phytic acid, increased chlortetracycline* serum levels when this antibiotic was fed to rats. Excess calcium and magnesium depressed chlortetracycline blood levels. The theory was advanced that the compounds that increase chlortetracycline serum levels function by suppressing the ionization of calcium, which inhibits chlortetracycline absorption from the intestine. Subsequently, Kiser² showed that a simple reduction in the calcium level of the diet gave a marked increase in chlortetracycline blood level in chicks and that ethylenediamine tetraacetic acid, which chelates calcium, produced a further increase in chlortetracycline serum levels. Peterson³ reported that terephthalic acid gave a fourfold increase in chlortetracycline serum levels and a fourfold increase in therapeutic effect against cecal coccidiosis. It has also been observed by Peterson et al⁴ that adding terephthalic acid to the diet led to a twofold increase in the therapeutic effect of oxytetracycline and erythromycin.

In view of the importance of increasing the therapeutic effect in chicks of orally administered tetracycline antibiotics by increasing their intestinal absorption, it seemed of interest to study further the effect of calcium levels and various calcium salts.

METHODS

A low calcium diet was prepared which contained .35 per cent calcium, most of which was supplied by the 5 per cent fishmeal (table I). This diet is essentially a commercial type of diet from which the calcium carbonate and calcium phosphate have been omitted as customary sources of calcium and phosphorus. Barred Rock-New Hampshire cross chicks were kept in electrically heated cages and given feed and water *ad libitum*. In the short-term experiments, 6 birds, 25 days old, were placed on the experimental diets for three days. At the end of this time they were bled by heart puncture, and the blood samples were combined into two pools of 3 birds each. Chlortetracycline was assayed in the serum by the *Bacillus mycoides* cup-plate method⁵ for determination of tetracyclines in serum and body fluids.

RESULTS

A comparison of various levels and sources of calcium and phosphorus was made in a short-term feeding experiment with chlortetracycline. In this experiment the diet was supplemented with either calcium carbonate or calcium sulfate as sources

* The trade name of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin.

TABLE I
Composition of Diet

Ingredient	Amount, Gm./Kg.
Corn	650
Soybean oil meal	200
Corn gluten meal	50
Fish meal	50
Alfalfa meal	20
Distillers' solubles	25
Choline chloride	.25
Vitamin supplement*	1.5
Vitamin A and D supplement (10,000 units A and 2000 units D/Gm.)	1
Delamix† (trace mineral supplement) with manganese and zinc	1

* The vitamin supplement furnished 4 p.p.m. riboflavin, 8 p.p.m. calcium pantothenate, 18 p.p.m. niacin, and 10 µg. B₁₂ per Kg. of diet.

† Obtained from Limestone Products Co. This trace mineral mixture furnished 60 p.p.m. of manganese and 20 p.p.m. of zinc.

of calcium and with either phosphoric acid or calcium phosphate as sources of phosphorus. It was thought that a relatively insoluble calcium salt, such as the sulfate, might yield a lower calcium ion concentration and therefore minimize the interference of calcium with chlortetracycline absorption. Terephthalic acid and ethylenediamine tetraacetic acid were added at levels of 5 Gm./Kg. of diet, and 200 p.p.m. of chlortetracycline was added to the diet of all groups. Two experiments were carried out. The results, which appear in table II, show that the low calcium diet gave a chlortetracycline serum level of 0.52 µg./ml. in two experi-

TABLE II
Effect of Various Calcium and Phosphorus Supplements and of Adjuvants on Chlortetracycline Blood Levels*

Source of calcium	Source of phosphorus	Chlortetracycline serum levels, µg./ml.								
		Control			0.5 per cent TPA†			0.5 per cent EDTA‡		
		Exp. 1	Exp. 2	Av.	Exp. 1	Exp. 2	Av.	Exp. 1	Exp. 2	Av.
—	—	.52	.52	.52	.95	1.12	1.03	.57	.57	.57
Calcium sulfate	—	.28	.26	.27	.66	.64	.65	.39	.39	.39
Calcium sulfate	Phosphoric acid	.35	.35	.35	.62	.70	.66	.33	.44	.39
Calcium sulfate	Calcium phosphate	.17	.19	.18	.38	.42	.40	.23	.33	.28
Calcium carbonate	—	.19	.13	.16	.36	.35	.36	.18	.25	.22
Calcium carbonate	Phosphoric acid	.12	.07	.10	.16	.26	.21	.28	.22	.25
Calcium carbonate	Calcium phosphate	.09	.09	.09	.23	.20	.22	.17	.19	.18

* Supplements add 1.0 per cent calcium and 0.2 per cent phosphorus.

† Terephthalic acid.

‡ Ethylenediamine tetraacetate.

ments. The addition of calcium sulfate to the diet led to a 50 per cent reduction in chlortetracycline serum level, while a combination of calcium sulfate and phosphoric acid produced a higher level than calcium sulfate alone. The addition of calcium phosphate to calcium sulfate caused a marked drop in serum level. Thus the addition of phosphoric acid gave a higher chlortetracycline serum level than the addition of calcium phosphate to a diet containing calcium sulfate. The addition of calcium carbonate, without an added phosphorous supplement, caused a larger drop in chlortetracycline level than the addition of calcium sulfate. The further addition of either phosphoric acid or calcium phosphate caused a further decrease. It is interesting to note that the addition of phosphoric acid to a diet containing added calcium carbonate caused a 60 per cent decrease in chlortetracycline serum level, whereas the addition of phosphoric acid to a calcium sulfate supplement produced a 23 per cent increase.

The data also show that the addition of terephthalic acid at a level of 0.5 per cent produced a twofold increase in chlortetracycline serum level on all types of diets. The highest serum level was obtained by the addition of 0.5 per cent terephthalic acid to a low calcium diet. It is also interesting that a diet with calcium sulfate and phosphoric acid but without terephthalic acid gave a higher chlortetracycline blood level than a conventional diet containing calcium carbonate and calcium phosphate supplemented with terephthalic acid.

The addition of ethylenediamine tetraacetate also produced increases in chlortetracycline serum levels. However, it had only a small augmenting effect on the low calcium diet, and such a small effect might be expected if ethylenediamine tetraacetate were functioning only by the chelation of calcium. The effect of this agent seemed most marked on diets that contained calcium phosphate together either with calcium carbonate or calcium sulfate.

A comparison was made of the effects of various calcium salts on chlortetracycline serum levels in short-term experiments from day 28 to day 31. Sodium phosphate was used as the phosphorus source in this experiment, and all diets contained 200 p.p.m. of chlortetracycline. The calcium salts were added to give an additional 0.6 per cent calcium, making a total of 1.0 per cent calcium. The results of this experiment, presented in table III, show that calcium carbonate, cal-

TABLE III

Effect of Various Calcium Salts on Chlortetracycline Blood Serum Levels

Supplement	Chlortetracycline, μg./ml.	Relative level, μg./ml.
None (0.4 per cent calcium)	0.29	2.5
Calcium carbonate	0.11	1.0
Calcium oxide	0.10	0.9
Calcium chloride	0.11	1.0
Calcium sulfate	0.18	1.6
Calcium gluconate	0.29	2.6
Calcium lactate	0.21	1.9
Calcium carbonate plus sodium sulfate	0.16	1.5
Calcium sulfate plus sodium carbonate	0.19	1.7
Calcium carbonate plus sulfuric acid	0.20	1.8

TABLE IV

Effect of Levels of Calcium Salts on Chlortetracycline Serum Levels and on Water Consumption at 8 Weeks

Calcium in diet, per cent	Added as calcium salt, per cent	Water consumption, (Gm./bird/day)		Chlortetracycline in serum, µg./ml.	
		Calcium sulfate	Calcium carbonate	Calcium sulfate	Calcium carbonate
0.55	0.20	166	158	0.56	0.51
0.75	0.40	168	139	0.46	0.26
1.10	0.75	212	148	0.31	0.17
1.52	1.17	250	154	0.29	0.06
2.03	1.68	306	150	0.25	0.04

cium oxide, and calcium chloride all markedly depressed the chlortetracycline serum level when added to a low calcium diet. Calcium gluconate produced no depression in chlortetracycline levels, while calcium lactate and calcium sulfate gave partial depression of chlortetracycline serum levels. It is also interesting to note that the addition of equivalent amounts of sodium sulfate to the calcium carbonate gave the same results as calcium sulfate alone. The addition of sodium carbonate to calcium sulfate did not depress chlortetracycline absorption.

The effect of different levels of calcium salts on chlortetracycline absorption and water consumption was studied in a continuous eight week feeding experiment. Duplicate groups of 12 birds were used for each treatment. Chlortetracycline serum levels were determined on 10 birds in each treatment. The water consumption figures are corrected for evaporation from the water troughs. The results, which appear in table IV, show that at calcium levels of 0.55 per cent the two calcium salts gave essentially the same chlortetracycline levels. However, increasing the calcium level to 2.03 per cent with the sulfate produced a 55 per cent reduction, while the carbonate produced a 92 per cent reduction. It is also apparent that increasing quantities of the carbonate had no effect on water consumption, while increasing quantities of the sulfate produced a marked increase. This could produce diarrhea and wet litter when large quantities of the sulfate are fed.

SUMMARY

1. A study was made of the effect of levels of calcium and of various sources of calcium and phosphorus on the serum levels of birds fed chlortetracycline.
2. A low calcium diet gave the highest chlortetracycline serum level. Increasing the calcium level decreased the antibiotic absorption. Calcium sulfate in the diet gave higher chlortetracycline serum levels than calcium carbonate.
3. The use of phosphoric acid as a source of phosphorus gives higher chlortetracycline serum levels than calcium phosphate when calcium sulfate is used as the calcium source.
4. The addition of 0.5 per cent terephthalic acid gave approximately a twofold increase in chlortetracycline serum level on all the diets used. Ethylenediamine

tetraacetic acid increased chlortetracycline absorption but was less effective than terephthalic acid.

5. Increasing amounts of calcium sulfate increased water consumption in birds, while increasing amounts of calcium carbonate had no effect.

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Some Observations on the Treatment of Tuberculosis in Infants and Children with Kanamycin and Isoniazid

ROBERT H. HIGH

*St. Christopher's Hospital for Children, the Department of Pediatrics,
Temple University School of Medicine, Philadelphia, Pa.*

Kanamycin, an antibiotic substance isolated as a fermentation product of *Streptomyces kanamyceticus* in 1957 by Umezawa et al,¹⁻³ was shown to have in vitro and in vivo activity against *Mycobacterium tuberculosis*.⁴ Animal experimentation⁵ and clinical trials⁶ in human subjects indicated that kanamycin was not so active as streptomycin in tuberculous infections. Moreover, the daily intramuscular administration of this agent to tuberculous adults for periods longer than 30 days or in amounts greater than 20 Gm. was associated with a distressingly high frequency of permanent nerve deafness. Similar findings have been made in adults treated with kanamycin for other infections.⁷ This toxic reaction is especially common in adults with poor renal function.

Observations made on infants and children⁸ during and following the administration of kanamycin by the intramuscular route for the treatment of a variety of infections suggested that its toxic reactions, especially ototoxicity, were considerably less frequent in children than in adults.

Treatment of various types of tuberculosis in infants and children employing kanamycin concomitantly with isoniazid seemed indicated to determine the effectiveness and toxicity of such combined antimicrobial therapy. The need for such trials is supported by the recent report of Debré et al⁹ who noted an increasing number of fresh tuberculous infections in infants and children caused by streptomycin-resistant *Myco. tuberculosis*. Such infections are unusual in this country.¹⁰

MATERIAL AND METHODS

Between February, 1958, and September 30, 1959, 22 infants and children ranging in age from 5 months to 11 years with various forms of tuberculosis were treated concurrently with kanamycin intramuscularly and isoniazid by mouth. A few observations have previously been reported on 7 of these 22 patients.⁸ Kanamycin and isoniazid were administered to 21 patients during hospitalization at St. Christopher's Hospital for Children and to the remaining patient during hospitalization at Philadelphia General Hospital, Northern Division. Some patients continued to receive kanamycin after discharge from the hospital. The patients received isoniazid after discharge for periods up to 18 months. It is planned to treat all patients with isoniazid for a period of at least one year. Pertinent details concerning these patients are listed in table I.

The diagnosis of tuberculosis was regarded as likely if the first or intermediate purified protein derivative tests, using 0.00002 or 0.0001 mg. of purified protein derivative intradermally, respectively, was positive and the clinical findings were

compatible with this diagnosis. In addition, two or more of the following criteria were present in all except patients 2 and 6: positive culture for *Myco. tuberculosis*, roentgenographic abnormalities compatible with tuberculosis, home contact with a known case of tuberculosis and, where applicable, histopathological changes compatible with the diagnosis of tuberculosis. In addition, most patients had negative intradermal histoplasmin tests. The diagnostic criteria are summarized in table II. Inspection of the table reveals that positive cultures were obtained in 9 patients and that histopathological studies were compatible with the diagnosis of tuberculosis in 8 patients, 3 of whom also had positive cultures for *Myco. tuberculosis*. In the remaining 8 patients, the diagnosis of tuberculosis cannot be conclusively established but is regarded as the most likely cause for the patients' disease from the available data.

Various observations including examination of blood counts, urinalysis, urea nitrogen content of the blood, and the patient's auditory function were made at various times before, during, and, in some instances, after the course of treatment with kanamycin. Bronchoscopic examinations were performed on one occasion in 11 patients. Six of these patients were subsequently examined two or more times. A total of 28 bronchoscopic observations were made.

Since the dose of kanamycin intramuscularly in the treatment of tuberculosis in children is not established, a wide variety of doses were employed ranging from 8 to 90 mg./Kg./day. Most patients received kanamycin in doses ranging from 10 to 25 mg./Kg./day. Kanamycin was administered daily in 21 patients for periods ranging from two weeks to eight months. In addition, 14 of these patients subse-

TABLE I

Infants and Children Treated with Kanamycin and Isoniazid According to Type of Tuberculosis

Patient	Age	Race	Sex	Weight, Kg.	Type of tuberculosis
1	5 mo.	N	F	7	Pulmonary
2	5 mo.	N	F	6.5	Pulmonary
3	8 mo.	N	M	8	Pulmonary
4	8 mo.	W	M	8	Pulmonary
5	12 mo.	W	M	11	Pulmonary
6	16 mo.	W	F	10	Pulmonary
7	2½ yr.	N	F	13	Pulmonary
8	9 yr.	N	F	25	Pulmonary
9	9½ yr.	N	M	26	Pulmonary
10	11 yr.	N	M	35	Pulmonary
11	14 mo.	W	F	10	Adenitis
12	23 mo.	N	F	12	Adenitis
13	2½ yr.	N	F	16	Adenitis
14	2½ yr.	N	F	14	Adenitis
15	9 yr.	N	F	31	Adenitis
16	10 yr.	N	M	35	Adenitis
17	15 mo.	W	F	9	Meningitis
18	16 mo.	W	F	10	Meningitis
19	7 yr.	N	F	17	Meningitis and miliary
20	7 mo.	N	F	6	Miliary and pulmonary
21	7 yr.	N	F	30	Pleural effusion
22	11 yr.	N	M	27	Psoas abscess and Pott's disease

TABLE II

*Tuberculosis in Infants and Children Treated with Kanamycin
and Isoniazid According to Diagnostic Criteria*

Patient	Positive purified protein derivative	Positive culture	Compatible roentgenograms	Home contact	Compatible histopathology
1	+	+	+	+	Not applicable
2	+	Pending	+	0	Not applicable
3	+	0	+	+	Not applicable
4	+	+	+	+	Not applicable
5	+	0	+	+	Not applicable
6	+	0	+	0	Not applicable
7	+	0	+	+	Not applicable
8	+	+	+	+	Not applicable
9	+	Pending	+	+	Not applicable
10	+	0	+	+	Not applicable
11	+	+	0	0	+
12	+	0	0	?	+
13	+	0	0	+	+
14	+	0	0	+	+
15	+	+	+	+	+
16	+	0	+	+	+
17	+	+	+	+	Not applicable
18	+	+	0	+	Not applicable
19	+	+	+	+	Not applicable
20	+	0	+	+	Not applicable
21	+	0	+	0	+
22	+	+	+	0	+

quently received kanamycin twice weekly for periods of four weeks to six months. One patient received kanamycin twice weekly for one month. All patients received isoniazid by mouth concurrently with the administration of kanamycin. The daily dose of isoniazid ranged from 7 to 40 mg./Kg., although in most instances it approximated 10 to 20 mg./Kg. The details of treatment for each patient are shown in table III.

Seventeen patients never received antituberculous therapy prior to the present study. Two had received significant amounts of antimicrobial treatment although patient 15 had not received such agents in the preceding three years. The remaining three patients, 4, 17, and 18, had been treated for tuberculosis for short periods prior to the institution of the present form of therapy.

In addition to antimicrobial therapy, 4 patients with severe bronchial obstruction secondary to massive lymphadenopathy also were treated with prednisone. The same agent was employed in the patient with the tuberculous pleural effusion and in the 3 patients with tuberculous meningitis.

All patients with tuberculous adenitis were operated upon and as many involved nodes as practical were removed. The patient with the tuberculous psoas abscess underwent surgical drainage. One patient with obstruction to the flow of spinal fluid secondary to tuberculous meningitis had a subarachnoid-peritoneal shunt performed to relieve the obstruction.

RESULTS

The current status of 18 patients has been recently ascertained and is shown in table IV. All but one is doing well, showing evidence of healing or arrested tuber-

culosis. One patient treated for tuberculous meningitis has arrested meningitis but shows devastating neurological residua. Four patients have moved from the area and have not been evaluated recently. They are, however, known to be living 10 to 14 months after their last evaluation.

All patients had urinalyses performed prior to the institution of treatment with kanamycin and isoniazid. Twenty-one patients had urinalyses performed during the

TABLE III
*Tuberculosis in Infants and Children Treated with Kanamycin
and Isoniazid According to Dosage Schedules*

Patient	Previous treatment		Current treatment, mg./Kg./day			
	Drugs	Duration	Kanamycin		Isoniazid	
			Dose	Duration	Dose	Duration
1	None		10	21 weeks	40	21 weeks
2	None		20	13 weeks	10	13 weeks
3	None		25	6 months	20	8 months
4	Streptomycin, isoniazid	1 month	20	1 month	9	1 month
5	None		28	6 weeks	9	16 months
			14	10 weeks		
6	None		20*	1 month	10	1 year
7	None		40	2 weeks	10	16 months
			20	5 weeks		
			20*	2 months		
8	Streptomycin, <i>p</i> -aminosalicylic acid, isoniazid, cycloserine	Irregularly for four years	20	5 weeks	10	12 weeks
			25*	7 weeks		
9	None		19	4 weeks	19	11 weeks
			19*	7 weeks		
10	None		15	7 weeks	8	21 weeks
			15*	10 weeks		
11	None		10	9 weeks	10	15 months
			10*	10 weeks		
12	None		23	4 weeks	8	12 months
			20*	6½ months		
13	None		30	3 weeks	15	13 weeks
			15*	10 weeks		
14	None		34	4 weeks	10	7 months
			34*	4 weeks		
15	Streptomycin, <i>p</i> -aminosalicylic acid, isoniazid	Several courses. None in preceding 3 years	25	2 weeks	7	7 months
			15*	6 months		
16	None		20	3 weeks	7	12 months
			15*	3 months		
17	Streptomycin, <i>p</i> -aminosalicylic acid, isoniazid	2 months	33	8 months	17	10 months
18	Streptomycin, isoniazid	2 weeks	20	10 weeks	20	17 weeks
			20*	7 weeks		
19	None		90	2 months	9	13 months
			22	1 month		
			22*	6 weeks		
20	None		25	6 months	25	8 months
21	None		8	3 weeks	7	4 months
			8*	11 weeks		
22	None		20	4 months	7	18 months
			10	1 month		
			10*	2 months		

* Single dose administered twice weekly.

TABLE IV
*Clinical Status of Tuberculous Infants and Children
Treated with Kanamycin and Isoniazid*

Patient	Status on discharge	Present status
1	Arrested	Living—details unknown
2	Still hospitalized	Healing
3	Still hospitalized	Healing
4	Improved	Living—details unknown
5	Healing	Apparently arrested
6	Improved	Living—details unknown
7	Improved	Apparently arrested
8	Same	Living—details unknown
9	Improved	Healing
10	Improved	Healing
11	Improved	Apparently arrested
12	Improved	Apparently arrested
13	Improved	Apparently arrested
14	Improved	Apparently arrested
15	Improved	Healing
16	Improved	Apparently arrested
17	Still hospitalized	Apparently arrested; severe residua
18	Arrested	Apparently arrested
19	Arrested	Apparently arrested
20	Still hospitalized	Healing
21	Healing	Healing
22	Healing	Apparently arrested

course of treatment because long-term administration of kanamycin is known to cause renal irritation. The number of studies per patient ranged from 1 to 34 and the total number of examinations was 263. In all but 3 patients urinalyses at some time showed the presence of small numbers of casts, red and white blood cells; in a few instances traces of albumin were noted. In no patient were these abnormalities so severe that kanamycin was discontinued.

Examination of the blood for the hemoglobin content, the white blood cell count, and the differential count were made prior to the start of treatment on all patients. Similar examinations were made during treatment in 19 patients. A total of 110 studies obtained during treatment failed to reveal any unusual findings other than the occurrence of eosinophilia ranging up to 6 per cent in 8 of the 19 patients examined.

The urea nitrogen concentration of the blood was determined in 13 patients before treatment was initiated and total of 47 such examinations were obtained in 9 patients during treatment. Abnormal values were not noted in any of the determinations including the 2 patients who developed some hearing loss.

Bronchoscopic examinations made prior to or during treatment revealed abnormalities compatible with the extent of the patients' disease.

Attention was directed at evaluating the hearing function in these 22 patients before, during, and, when possible, after the termination of treatment with kanamycin because of the known potential for ototoxicity following the intramuscular administration of this agent. These observations are presented in detail in table V. It was not possible to test all patients objectively but gross evaluations of hearing function were made on all patients before and during treatment. These findings were normal in all patients except patient 15 who was known to have hearing loss

TABLE V

*Auditory Function in Tuberculous Infants and Children Treated with
Kanamycin and Isoniazid in Relation to Kanamycin Treatment*

Patient	"Gross" observations			Audiometric studies		
	Pretreatment	Treatment	Post-treatment	Pretreatment	Treatment	Post-treatment
1	Normal	Normal	Not followed	Not done	Not done	Not done
2	Normal	Normal	Under treatment	Not done	1-PGSR* Normal	Under treatment
3	Normal	Normal	Normal	Not done	1-PGSR Normal	1-PGSR Normal
4	Normal	Normal	Not followed	Not done	Not done	Not done
5	Normal	Normal	Normal	Not done	Not done	Not done
6	Normal	Normal	Not followed	Not done	Not done	Not done
7	Normal	Normal	Normal	Not done	1-F.F.† Normal 1-PT‡ Normal	Not done
8	Normal	Normal	Not followed	Not done	Not done	Not done
9	Normal	Normal	Normal	Not done	3-PT Normal	Under treatment
10	Normal	Normal	Normal	Not done	2-PT Normal	Under treatment
11	Normal	Normal	Normal	Not done	Not done	Not done
12	Normal	Normal	Normal	Not done	Not done	Not done
13	Normal	Normal	Under treatment	Not done	Not done	Not done
14	Normal	Normal	Normal	Not done	1-PGSR Normal	Not done
15	Decreased	Decreased	Decreased	1-PT Decreased 20-40 Db.	1-PT Decreased 20-40 db.	Under treatment
16	Normal	Normal	Normal	1-PT Normal	1-PT Normal	1-PT Normal
17	Comatose	Comatose	Comatose	Not done	Not done	1-PGSR Some function
18	Comatose	Normal	Normal	Not done	1-PGSR Normal	Under treatment
19	Comatose	Normal	Normal	Not done	5-PT Bilateral loss at 6 and 8 k.	Not done
20	Normal	Normal	Normal	Not done	2-PGSR Normal then slight loss at 8 k.	1-PGSR Slight loss at 8 k.
21	Normal	Normal	Normal	1-PT Normal	2-PT Normal	1-PT Normal
22	Normal	Normal	Normal	Not done	6-PT Normal then tran- sient loss at 3, 4, 6 k. then normal	1-PT Normal

* PGSR, psychogalvanic stimulation reaction.

† F.F., free field test.

‡ PT, pure tone audiometry.

as a result of chronic, purulent otitis media and in patient 17 who could not be adequately evaluated because of severe neurological sequelae from tuberculous meningitis. Infants and younger children were observed for their continuing ability to respond appropriately to conversational tones and also for their ability to acquire new language skills. The latter two observations in infants and young children are fairly satisfactory indications of adequate hearing perception at most tones except those greater than 4000 cycles/sec. Although such observations are not so satisfactory as objective measurements of auditory function, they do permit limited interpretation of hearing perception. As shown in table V, no gross hearing abnormalities were noted in any patients except the 2 already mentioned.

Objective measurements of hearing were obtained on 14 patients. These are also tabulated in table V. It was possible to obtain 37 tests on these subjects. Infants and younger children were tested by the psychogalvanic stimulation response technique on nine occasions and by free field testing on one occasion. Children were tested by pure tone audiometry on 27 occasions. Pretreatment observations were made on only 3 patients but the first test obtained during treatment on patients 9, 10, and 22 were made before the completion of one month of treatment with kanamycin. It would seem important that the first examination made during treatment with kanamycin was regarded as normal in 10 of 13 patients tested objectively. Patient 15 with a preexisting hearing loss showed no worsening of her auditory acuity during the administration of kanamycin.

Two patients, 19 and 20, who received large amounts of kanamycin intramuscularly for the treatment of miliary tuberculosis associated with tuberculous meningitis and miliary tuberculosis respectively showed definite loss of high tone perception when first tested. The first patient received 99.2 Gm. (90 mg./Kg./day) of kanamycin over a period of 62 days before her hearing loss was detected. The second patient received 22.2 Gm. (25 mg./Kg./day) of kanamycin over a period of 148 days before her hearing loss was demonstrated. These 2 patients have shown no worsening of their hearing loss on subsequent tests. They have, however, shown no tendency for improvement.

DISCUSSION

Observations made during the course of treatment of a variety of types of tuberculosis employing kanamycin intramuscularly in combination with isoniazid in 22 infants and children have been presented. The number of patients studied is obviously too small to permit valid comparisons with the results of treatment using other combinations of antimicrobial agents. Such comparisons would require observations made on large numbers of infants and children with a variety of types of tuberculosis.

It should be recognized that a few of the patients included in the present group might have undergone spontaneous healing without antimicrobial therapy. Further, some patients included in this report could have achieved equally rapid improvement following the administration of isoniazid as the sole therapeutic agent. It is unlikely, however, that patients with tuberculous meningitis or miliary tuberculosis would become arrested without the administration of combined antimicrobial therapy. Such results would be unlikely following the use of isoniazid as the only anti-

microbial agent. The results noted in this group of patients, 17 to 20, would seem to indicate that the combination of kanamycin and isoniazid has value in the treatment of tuberculosis in infants and children. These limited studies cannot be interpreted to suggest that the programs of treatment of tuberculosis with other agents, especially with streptomycin and isoniazid, should be replaced by treatment with kanamycin and isoniazid.

The present plan of treatment might be employed in tuberculous infants and children who fail to respond to other agents. Therapeutic failures, especially when streptomycin and isoniazid have been administered, are distinctly uncommon in infants and children. If, however, fresh infections with streptomycin-resistant *Myco. tuberculosis* occur in this country in infants and children with increasing frequency as has been observed in France by Debré et al,⁹ the present plan of treatment might demand more consideration. Experience in this country indicates that such infections are relatively uncommon.¹⁰ In patients observed in our institutions, only one such fresh tuberculous infection produced by streptomycin-resistant *Myco. tuberculosis* has been observed since 1945.

The observations made on these patients have special interest in addition to considerations relative to the usefulness of a new combination of antituberculous agents. These patients, as shown in table III, received kanamycin in fairly large doses for relatively long periods. Accordingly, the observations of the toxicity of kanamycin under these circumstances have interest. Studies of renal toxicity as measured by abnormalities in the urinalyses and urea nitrogen content of the blood have failed to show any serious reactions. The presence of small amounts of albumin, casts, and cellular elements was observed in most patients. These abnormalities were never severe and never necessitated discontinuing treatment. Periodical examinations of the blood counts failed to show any significant change except for the occasional presence of eosinophilia, which did not exceed 6 per cent.

The studies of hearing function made on these patients have particular importance. The technical difficulty of adequately testing hearing in infants was such that all patients were not studied objectively. The observation that the infants continued to respond appropriately to conversational tones and that they acquired new language functions during the long-term administration of kanamycin strongly suggests that they had not suffered any significant hearing loss at least at tones less than 4000 cycles/sec.

The results of objective hearing tests suggest that these gross observations are probably valid. Although the number of patients tested was not large, more than half of the patients in this group were subjected to hearing tests. Only 2 of 13 patients with presumably normal hearing prior to the institution of kanamycin treatment were noted to have high tone hearing loss. These observations suggest that infants and children have less risk of hearing loss following the administration of kanamycin than has been observed in adults.

These findings may be of importance in considering the long-term administration of kanamycin to infants and children with infections other than tuberculosis.

SUMMARY

Observations have been made on 22 infants and children with a variety of types

of tuberculosis who were treated concurrently with kanamycin intramuscularly and isoniazid by mouth. These observations suggest the following: (1) Such antimicrobial treatment is likely to arrest the tuberculous infection. (2) This program of treatment will probably not supplant established therapeutic programs in tuberculous infants and children except under unusual circumstances. (3) The toxic reactions to kanamycin have not been severe. (4) Hearing loss in infants and children following the long-term administration of kanamycin is apparently less frequent than has been observed in adults.

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Erythromycin Propionate: A Clinical and Laboratory Study

S. SALITSKY, J. K. GEIGER, W. J. MELLMAN, AND E. L. FOLTZ
WITH THE TECHNICAL ASSISTANCE OF BETTY S. GRAVES

Department of Pediatrics and Section of Infectious Diseases, William Pepper Laboratory for Clinical Medicine, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Since the introduction of erythromycin in 1952, various modifications have been studied in an attempt to overcome its disadvantages of insolubility, bitter taste, and inactivation by gastric acid. It is obvious that a compound that is palatable and stable enough to be administered orally is highly desirable. Also a suspension is especially valuable in pediatric practice.

During the past few years a number of erythromycin esters have been evaluated.¹⁻³ These include the propionyl ester (Ilosone*), which has been reported to produce earlier, higher, and more prolonged blood concentrations than the erythromycin base,^{4,6} while maintaining low toxicity and allergenicity.^{7,8} Comparison studies with triacetyloleandomycin have shown that the propionyl form of erythromycin produces significantly higher serum levels.⁹⁻¹¹

The purpose of this study was threefold: first, to study the therapeutic effectiveness of erythromycin propionate against certain bacterial infections in children; second, to compare by a crossover study in adult males the serum levels after a single oral dose of erythromycin base and the propionyl ester; and finally, to determine the serum levels of erythromycin in children after a single dose of the capsule and lauryl sulfate suspension forms of propionyl erythromycin.

METHOD

Thirty-nine children seen in an outpatient department with a variety of infections, requiring antibiotics in the opinion of the examining physician, were given erythromycin propionate in capsule form according to the following dosage schedule: 50 lb. or less, 125 mg. every six hours; 51 to 100 lb., 250 mg. every eight hours; 100 lb. or more, 250 mg. every six hours.

Since the capsule form was used, it was generally reserved for children more than 5 years of age. An appropriate bacterial culture was obtained in all but 1. The patients were evaluated at the end of two and six days. The duration of therapy averaged six to eight days. A satisfactory response was considered to have occurred if the patient was afebrile within 48 hours and showed considerable improvement in his general condition.

Ten healthy adult males were studied by crossover techniques. On the first day, 5 of the subjects received the erythromycin base and the other 5 received the propionyl ester. Six days later the groups were reversed. In all cases a 250 mg. capsule of the specified form was given in the fasting state. Blood specimens were drawn prior to administration of the drug and at ½, 1, 2, 4, 6, 8, and 12 hours

* The trade name of Eli Lilly & Co. for the propionyl ester of erythromycin is Ilosone.

TABLE I

Clinical Results in Pediatric Infections with Erythromycin Propionate

Diagnosis	No. cases	Age, yr.	Predominating bacteria	Favorable response
Pharyngitis-tonsillitis	23	4-12	Hemolytic <i>Staph. aureus</i> coagulase +, 4 <i>a</i> <i>Streptococcus</i> , 8 <i>a'</i> <i>Streptococcus</i> , 3 <i>βStreptococcus</i> , 8	22/23
Otitis media, acute	8	2 1/2 10	<i>a</i> <i>Streptococcus</i> , 1 <i>a'</i> <i>Streptococcus</i> , 3 Hemolytic <i>Staph. aureus</i> coagulase +, 3 <i>βStreptococcus</i> , 1	6/8
Otitis media, chronic	1	10	<i>Proteus</i> , 1	0/1
Cervical adenitis	4	5-11	<i>a'</i> <i>Streptococcus</i> , 2 <i>a</i> <i>Streptococcus</i> , 1 <i>βStreptococcus</i> , 1	4/4
Inguinal adenitis	1	13	?	1/1
Pyoderma complicating varicella	1	7	Hemolytic <i>Staph. albus</i> , coagulase +	1/1
Infected atopic eczema*	1	5	Hemolytic <i>Staph. aureus</i> , coagulase + <i>Cl. perfringens</i>	1/1
Total	39			35/39

*Also treated with bacitracin locally.

after taking the drug. Sera were harvested and frozen immediately. Bioassay was carried out with a cup-plate method employing *Sarcina lutea* as the test organism.

Twelve children between the ages of 6 and 16 years received erythromycin propionate. A single 125 mg. or 250 mg. capsule was given according to the schedule used in the clinical trials. Feedings were not withheld.

Sixteen other children, 6 to 15 years of age, were given a single dose of the lauryl sulfate propionyl erythromycin suspension. Thirteen received 4.9 to 5.75 mg./lb. while the remaining 3 received 2.5 mg./lb. All 28 children received the drug from 30 to 60 minutes after breakfast. No difficulties in administering the suspension were encountered because of unpalatability.

None of these children had received antibiotics in the week prior to the test. Blood specimens were drawn at two, four, and six hours after the single oral dose. Concentrations were assayed as described previously.

RESULTS

The clinical results in the 39 children treated with erythromycin propionate are summarized in table I. The infections treated were primarily of three types: pharyngitis-tonsillitis, otitis media, and cervical adenitis. The study drug in capsule form was the only chemotherapeutic used, except in the 1 case where local bacitracin was employed.

Twenty-three patients were in the pharyngitis-tonsillitis group, with *Streptococcus* predominating in the nasopharyngeal culture in 19 and coagulase-positive *Staphylococcus* in 4. Satisfactory results were obtained in 22 of the 23. Nine children with otitis media were treated; 8 had acute and 1 had chronic disease. Bacterial isolates included streptococci in 5 and coagulase-positive staphylococci in 3 as the

predominating organism. Six of the 8 acutely ill patients responded favorably, while the patient with chronic disease due to *Proteus* did not. All 4 children with cervical adenitis responded satisfactorily, as did the 1 child with inguinal adenitis from which no culture was obtained. The 2 patients with pyoderma showed improvement and healing while on treatment.

Side effects were noted in 2 of the 39 (5 per cent). One patient with an allergic history had giant urticaria after the third dose, which disappeared rapidly with discontinuance of the drug. The other patient experienced nausea and vomiting after three doses, which likewise abated with termination of therapy.

The serum levels of the 10 subjects involved in the crossover study using erythromycin base and the ester are tabulated in table II. All levels are reported in $\mu\text{g./ml.}$ It is readily apparent that the propionyl ester produced serum levels that are significantly higher than those after administration of the base. This finding confirms previous reports.^{4,6}

Table III shows the serum levels obtained in the 12 children after a single oral dose of erythromycin propionate capsules. The patients can be subdivided into three groups based on dose related to body weight as follows: 1.4 to 1.7 mg./lb., 2.9 to 3.4 mg./lb., and 4.2 to 5.0 mg./lb. The average serum levels of these groups of 4, 3, and 5 children respectively are tabulated in table V.

TABLE II

Crossover Study of Serum Concentrations ($\mu\text{g./ml.}$) after 250 mg. of Erythromycin and Erythromycin Propionate Orally in Healthy Fasting Male Volunteers

Subject	Hours						
	1/2	1	2	4	6	8	12
<i>Erythromycin Base</i>							
Ca.	<0.066	<0.066	<0.066	<0.066	<0.066	<0.066	<0.066
Ch.	<0.066	0.13	<0.066	0.16	<0.066	<0.066	<0.066
Co.	<0.066	<0.066	<0.066	0.08	<0.066	0.08	<0.066
E.	<0.066	0.07	<0.066	0.26	0.13	0.07	<0.066
G.	<0.066	<0.066	<0.066	<0.066	<0.066	<0.066	<0.066
Kem.	<0.066	<0.066	<0.066	0.15	0.08	<0.066	<0.066
Ken.	0.16	<0.066	0.13	0.21	0.10	0.07	<0.066
P.	<0.066	<0.066	<0.066	0.07	<0.066	<0.066	<0.066
S.	<0.066	<0.066	<0.066	0.14	0.10	<0.066	<0.066
W.	0.07	<0.066	<0.066	0.09	<0.066	<0.066	<0.066
Average	0.02	0.02	0.01	0.12	0.04	0.02	<0.066
<i>Erythromycin Propionate</i>							
Ca.	<0.066	0.09	0.26	0.21	0.11	<0.066	<0.066
Ch.	<0.066	<0.066	0.28	0.60	0.35	0.22	0.11
Co.	<0.066	<0.066	0.10	0.41	0.26	0.13	0.07
E.	0.80	2.95	3.50	2.50	0.72	0.42	0.25
G.	<0.066	0.10	0.24	0.38	0.29	0.20	0.10
Kem.	<0.066	<0.066	0.22	2.05	0.52	0.35	0.16
Ken.	<0.066	<0.066	0.17	0.14	0.09	<0.066	<0.066
P.	<0.066	<0.066	0.51	0.30	0.17	0.10	0.07
S.	0.10	3.90	2.60	2.50	0.76	0.50	0.23
W.	<0.066	0.42	3.05	2.05	0.58	0.42	0.17
Average	0.09	0.74	1.09	1.11	0.38	0.23	0.12

TABLE III

Serum Concentrations ($\mu\text{g./ml.}$) in Pediatric Patients after a Single Oral Dose of Erythromycin Propionate Capsules

Subject	Age, yr.	Weight, lb.	Dose, mg./lb.	Hours		
				2	4	6
J. DeL.	13	170	1.47	0.085	3.0	2.75
S. M.	14	149	1.7	6.3	3.5	1.7
V. M.	12	147	1.7	0.215	0.052	0.052
D. B.	16	160	1.6	0.39	0.19	0.155
B. B.	10	73	3.4	0.068	0.12	0.064
P. Y.	8	40	3.1	0.39	0.255	0.105
K. McN.	6	43	2.9	0.7	0.44	0.25
R. M.	13	104	4.8	0.26	1.25	1.35
L. M.	8	53	4.7	0.059	0.72	0.36
J. M.	7	50	5.0	0.12	0.071	0.06
D. T.	7	60	4.2	0.365	0.21	0.076
A. P.	15	77	4.9	1.7	5.8	4.1
Average				0.89	0.13	0.09

Table IV lists the serum levels of 16 children given a single oral dose of the erythromycin propionate lauryl sulfate suspension. Thirteen received approximately 5 mg./lb. while the other 3 received half the dose. The average levels with these two dosage schedules are included in table V.

DISCUSSION

The clinical trials with erythromycin propionate suggest the usefulness of this agent; with the infections caused by gram-positive cocci, satisfactory responses were

TABLE IV

Serum Concentrations ($\mu\text{g./ml.}$) in Pediatric Patients after a Single Oral Dose of Lauryl Sulfate Erythromycin Propionate Suspension

Subject	Age, yr.	Weight, lb.	Dose, mg./lb.	Hours		
				2	4	6
G. P.	11	68	5.5	4.4	2.8	1.35
C. D.	11	87	5.75	4.8	2.95	2.45
C. A.	11	52	4.8	4.0	3.9	3.1
R. F.	10	70	5.35	3.8	3.9	3.0
A. P.	15	77	4.9	5.0	6.5	4.0
W. J.	7	75	5.0	3.8	3.2	0.64
K. T.	7	45	5.0	0.53	0.85	0.19
I. J.	9	50	5.0	0.66	0.40	0.48
D. H.	6	49	5.1	3.6	3.7	2.0
D. K.	11	82	4.9	3.6	2.7	0.72
A. R.	8	68	5.2	6.0	4.2	5.2
D. C.	6	40	5.0	3.4	2.65	1.35
D. R.	8	59	5.1	3.3	2.45	1.1
E. P.	9	56	2.5	3.0	2.05	0.235
M. J.	6	60	2.5	3.3	3.5	3.2
P. VanL.	15	160	2.5	2.35	1.1	0.53
Average				3.47	2.93	1.73

TABLE V

*Average Serum Concentrations ($\mu\text{g./ml.}$) after Various Dosage Levels of
Erythromycin Propionate*

Number of cases	Dosage range, mg./lb.	Hours			
		2	4	6	
<i>Capsules</i>					
4	1.5-1.7	1.75 (0.23)	1.81 1.25	1.16 0.99)	excluding S. M., table III
3	2.9-3.4	0.39	0.27	0.14	
5	4.2-5.0	0.50 (0.20)	1.61 0.56	0.91 0.46)	excluding A. P., table III
<i>Lauryl Sulfate Suspension</i>					
13	4.8-5.8	3.60	3.09	1.94	
3	2.5	2.88	2.22	1.66	

obtained in 90 per cent of the children treated. Since a fall in the temperature was recorded within the first 48 hours of treatment in these cases, the antibiotic may have contributed in the management of these problems by inhibition of the bacterial components. The study is lacking in untreated controls to show the definitive action of the antibiotic, but experience with simple antipyretics would suggest that the other manifestations of infection were favorably influenced by the antimicrobial therapy.

Absorption of erythromycin propionate in capsules in the children studied (table III) was somewhat less than that observed in adults (table II); the adult data confirm previous reports. In this study children—not adults—were used, and the subjects were given the drug in the nonfasting state. This suggests that the postabsorptive state or increased acid secretion of the stomach affects the absorptive efficiency of this form of erythromycin. We have no explanation for the unusually high serum levels of 2 children (S. M., A. P.). Severe anemia in A. P. may have contributed to the unusual results.

The striking increases in serum concentrations when the lauryl sulfate modification was given under similar conditions as the simple propionyl ester tend to corroborate in vitro data indicating that this drug is stable in gastric acid.¹³ This property appears to obviate the need for the fasting state to attain optimal drug absorption.

Table V suggests that there is no correlation between dosage level in terms of body weight of either suspension or capsule of erythromycin propionate and serum concentrations of erythromycin. The optimal dosage of the drug may have been exceeded in many of these patients, so that the percentage of drug absorption decreases as additional drug is ingested. Additional pharmacological information is needed to resolve this.

There is no close correlation between the serum concentration and clinical response. Because of the nature of the illnesses studied, there is no proof that the bacterial isolates were the primary cause of disease; viral agents were undoubtedly

operative in some of the patients treated. However, bacterial complications or recurrences did not occur in the treated cases. In these circumstances high serum concentrations may not be necessary for therapeutic efficacy, while in more serious or "deep-seated" infections the attainment of maximum serum concentration may be critical.

CONCLUSIONS

1. Good clinical responses with erythromycin propionate were obtained in 90 per cent of 39 children treated with infections in a hospital outpatient department.
2. In a crossover study of 10 healthy male subjects, significantly higher and earlier rises in serum concentrations of erythromycin were obtained when erythromycin propionate was compared to erythromycin base. The subjects were fasted during the entire study.
3. Comparable serum levels were not observed when the erythromycin propionate was administered to a group of nonfasted children.
4. The lauryl sulfate modification of erythromycin propionate in suspension produced significantly higher levels in a similar group of nonfasted children, comparable to these obtained in the fasted adults given unmodified erythromycin propionate.

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Clinical Studies of Propionyl Erythromycin Lauryl Sulfate in Children

THOMAS E. REICHELDERFER, ROBERT L. BAIRD, AND HELEN J. OSSOFKY

Department of Pediatrics, District of Columbia General Hospital, Washington, D. C.

Erythromycin, an antibiotic with few side effects, was first isolated by McGuire et al¹ from the actinomycete *Streptomyces erythreus* in 1952. Since then it has found wide general acceptance. It is effective against gram-positive bacteria, gram-negative bacteria, large viruses, rickettsiae, spirochetes, and a few protozoa. Several clinical reports have established the usefulness of erythromycin base and its related compounds.²⁻⁴ The pharmacology, toxicity, and activity of propionyl erythromycin* were described in 1958.⁵ While propionyl erythromycin was similar to erythromycin base in many respects, it was reported that the propionyl ester of erythromycin, when ingested in a fasting state, produced higher and more prolonged serum levels.⁶⁻⁸ The erythromycin salts, erythromycin base, and erythromycin ester, however, because of a bitter taste in aqueous suspension, were not acceptable to children except in the form of capsules or enteric-coated tablets. The difficulty in obtaining an oral dosage form that would give consistent blood levels in spite of inactivation by gastric secretions was also inherent in the propionyl ester of erythromycin.

Stephens et al⁹ have described the preparation of a new erythromycin compound, propionyl erythromycin lauryl sulfate.* Propionyl erythromycin lauryl sulfate is prepared from erythromycin by esterification, followed by a double decomposition reaction with sodium lauryl sulfate. This new erythromycin derivative has an extremely low water solubility. It is practically tasteless and, therefore, may be formulated into a pleasantly flavored aqueous suspension. Gastric acidity is not strong enough to displace the acid radical of the salt of the strong acid (lauryl sulfuric acid), so that propionyl erythromycin lauryl sulfate remains undissolved in the gastric juice and retains its potency for several hours. It is probably absorbed in the un-ionized state and appears to give longer effectual blood levels.

Griffith¹⁰ had demonstrated that with a single dose of 5 mg./lb. of body weight of the aqueous suspension of propionyl erythromycin lauryl sulfate, high peak blood levels were obtained in children within two hours of ingestion, and therapeutic levels were still present six hours later.

In this study, an attempt was made to determine the optimum dosage of propionyl erythromycin lauryl sulfate that would give satisfactory blood levels. The therapeutic effectiveness of different dosages of the antibiotic in sick children was also undertaken, and effects of the administration of large doses of propionyl erythromycin lauryl sulfate in newborn and premature infants were also studied.

METHOD

An aqueous suspension of propionyl erythromycin lauryl sulfate containing sugars and flavoring agents for taste was given to all subjects.

Ten children between the ages of 7 months and 7 years who were hospitalized for surgical and medical conditions were given propionyl erythromycin lauryl sul-

* The trade name of Eli Lilly & Co. for propionyl erythromycin is Ilosone; for propionyl erythromycin lauryl sulfate, Ilosone lauryl sulfate.

fate. Two were given single doses of 5 mg./lb.; 4, 2.5 mg./lb.; and 4, 1.25 mg./lb. A crossover study of serum levels was undertaken in the fasting and nonfasting states. Blood was drawn at one, two, and six hours after the medication had been received, the blood was allowed to clot, and the serum was separated from it. The serum was frozen and stored at -20°C .

Levels of propionyl erythromycin lauryl sulfate were determined by serial tube dilutions of serum with beta-hemolytic *Streptococcus* (C203) in brain infusion broth by the Walter Reed Army Hospital modification of Rammelkamp's assay test in the laboratory of Dr. M. J. Romansky. The levels are reported in $\mu\text{g./ml}$.

Ten children who ranged in age from 5 months to 7 years and who were ill with

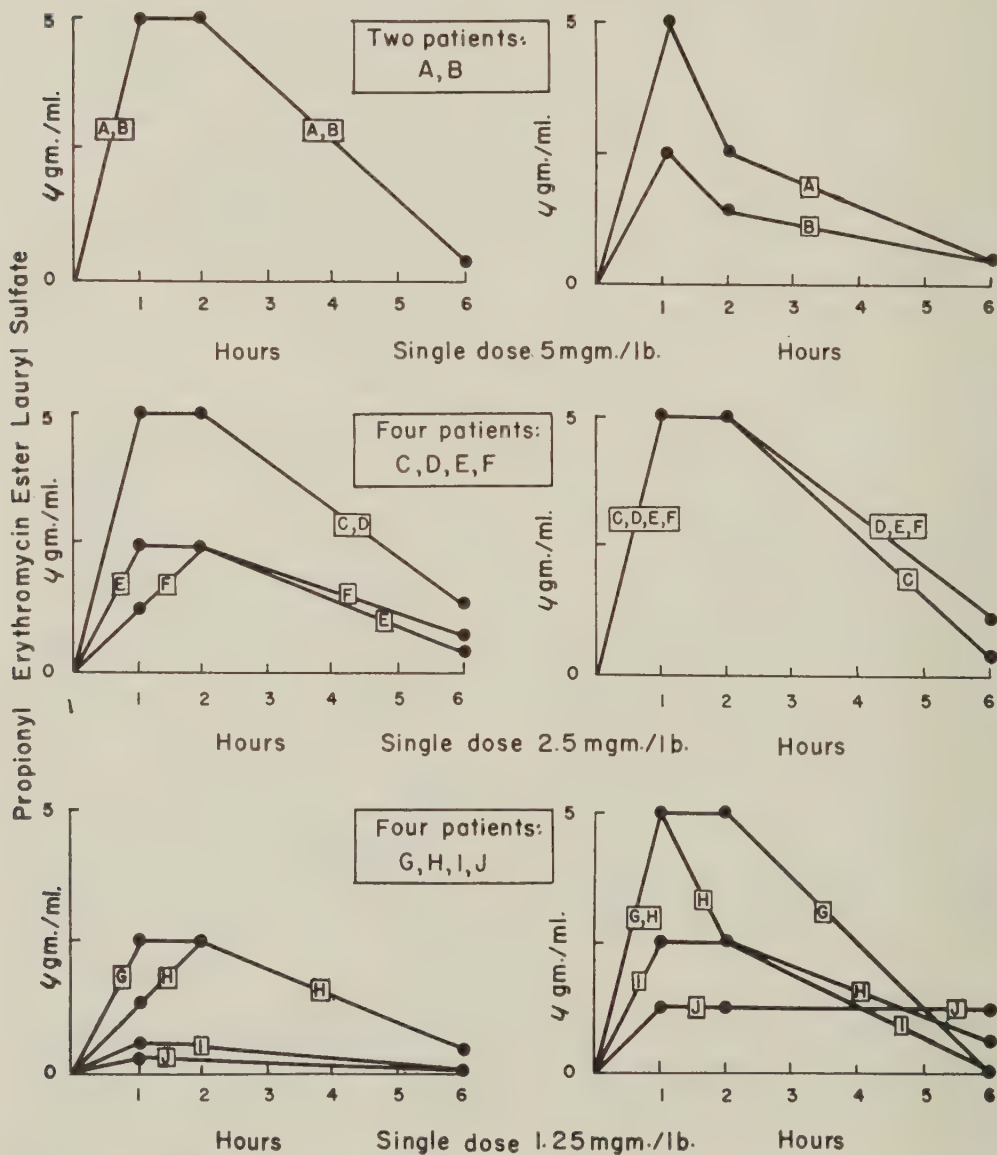


FIG. 1. Serum levels with varying single oral doses of propionyl erythromycin lauryl sulfate, left hand column in the nonfasting state, right hand column in the fasting state.

TABLE I

Clinical Results after Propionyl Erythromycin Lauryl Sulfate Therapy, 5 mg./lb./day

Subject	Sex	Race	Age	Diagnosis	Bacteriology	Durat., days	Results
D. M.	F	N	17 mo.	Bronchopneumonia	<i>Diplococcus pneumoniae</i> <i>Hemophilus influenzae</i>	5	Recovered
T. T.	M	N	5 mo.	Bronchopneumonia	<i>D. pneumoniae</i> , hemo- lytic <i>Staph. aureus</i>	4	Recovered
N. C.	F	N	2½ yr.	Pneumonia, right upper lobe	<i>D. pneumoniae</i>	5	Recovered
J. F.	M	N	19 mo.	Bronchopneumonia	<i>D. pneumoniae</i>	4	Recovered
J. McG.	M	N	2 yr.	Pneumonia, right upper lobe	<i>D. pneumoniae</i>	9	Improved
M. M.	M	C	7 yr.	Pneumonia, right upper lobe	Hemolytic <i>Staph. aureus</i>	8	Improved
W. N.	M	N	7 yr.	Pertussis bronchopneumonia	—	7	Improved
R. W.	M	N	4 yr.	Bronchopneumonia	<i>D. pneumoniae</i>	20	No effect
L. R.	F	N	1½ yr.	Pneumonia, left lower lobe	<i>D. pneumoniae</i>	5	No effect
W. D.	M	N	2 yr.	Pneumonia, right lower lobe	Hemolytic <i>Staph. aureus</i>	49	No effect

pneumonia received 5 mg./lb. of propionyl erythromycin lauryl sulfate per day and were evaluated again at the end of the fifth day of treatment. Ten additional children who ranged in age from 2 months to 4 years and who had respiratory illnesses and a *Staphylococcus* infection received 20 mg./lb. of body weight. They were also re-evaluated on the fifth day of therapy. All but one child had radiographic evidence of pneumonia.

Because of the reports of Sutherland¹¹ and Dorn and Smith¹² on the toxic effects and high cumulative blood levels of chloramphenicol in newborn infants, the effect of large dosages of propionyl erythromycin lauryl sulfate in a group of children was studied. Eleven newborn infants, 4 of whom were premature, received 100 mg. of propionyl erythromycin lauryl sulfate per Kg. per day for three to six days as prophylaxis against infections. One infant received 190 mg./Kg. for three days. Six were delivered by Caesarean section and the remainder were vaginal deliveries. Serum blood levels were determined on 2 premature infants after five days of treatment.

RESULTS

The serum levels after varying single oral doses of propionyl erythromycin lauryl sulfate in the fasting and nonfasting state are shown graphically in figure 1. It will be noted that the serum levels of 5 µg./ml. were more frequent when the dose of the drug exceeds 2.5 mg./lb. of body weight. Two children achieved a serum blood level of 5 µg./ml. with as little as 1.25 mg./lb. of body weight. The serum levels with this smaller dose were most irregular. The serum levels with a dose of 1.25 mg./lb. were within the range reported by Sylvester and Josselyn¹³ with slightly larger doses of erythromycin stearate. These authors were unable to get higher blood levels of erythromycin by doubling the dosage of the antibiotic. The high serum levels of erythromycin in this study exceeded by 2.5 times those reported for propionyl erythromycin ester by Griffith et al⁶ in adults. The serum levels of propionyl erythromycin lauryl sulfate rose to a higher level at two hours and remained in the range of the in vitro sensitivity of most susceptible microorganisms

TABLE II

Clinical Results after Propionyl Erythromycin Lauryl Sulfate Therapy, 20 mg./lb./day

Subject	Sex	Race	Age	Diagnosis	Bacteriology	Durat., days	Results
D. M.	M	C	2 mo.	Furunculosis	Hemolytic <i>Staph. aureus</i>	7	Recovered
A. C.	M	C	7 mo.	Bronchopneumonia	Hemolytic <i>Staph. aureus</i>	6	Recovered
D. J.	F	C	22 mo.	Bronchopneumonia	Hemolytic <i>Staph. aureus</i>	10	Recovered
J. H.	F	C	14 mo.	Pneumonia, right upper lobe, iron deficiency anemia	Alpha hemolytic streptococci	9	Recovered
J. L.	M	C	3 yr.	Bronchopneumonia	—	4	Recovered
B. N.	M	C	13 mo.	Pneumonia	Alpha hemolytic <i>Streptococcus</i>	3	Recovered
R. C.	M	C	1 yr.	Pneumonia, left upper lobe	Alpha hemolytic <i>Streptococcus</i>	7	Recovered
B. W.	M	C	2 yr.	Pneumonia, left lower lobe	<i>D. pneumoniae</i>	5	Recovered
S. H.	F	C	2 yr.	Asthmatic bronchopneumonia	Hemolytic <i>Staph. aureus</i>	8	Improved
E. D.	M	C	4 yr.	Pneumonia, right middle lobe	Hemolytic <i>Staph. aureus</i>	8	Improved

at the end of six hours. Food did not interfere to any great extent with the absorption of the antibiotic from the intestinal tract. The serum levels with the 5 mg./lb. dosage were within the range reported by Griffith.¹⁰

When propionyl erythromycin lauryl sulfate was given to 10 children with respiratory disease in the dosage of 5 mg./lb./day in four doses, 7 of the 10 showed a satisfactory clinical response within five days after the initiation of treatment (table I). Three of these children failed to respond to treatment with propionyl erythromycin lauryl sulfate. Two were known to have a microorganism that was susceptible to erythromycin and that caused their illness. One had underlying intrinsic pulmonary disease. Despite the small dosage, the majority of patients studied had a satisfactory clinical response.

The dosage of propionyl erythromycin lauryl sulfate was increased to 20 mg./lb./day in four doses in 10 children with furunculosis and respiratory disease. They were also evaluated on the fifth day of treatment (table II). All showed a prompt recovery from the illness. The larger dosage of the drug would appear to be more effective in the small group studied.

The large dosages of propionyl erythromycin lauryl sulfate that were given to newborn and premature infants had no adverse effect on them. They took their feedings well and thrived on the antibiotic. No vomiting, diarrhea, cyanosis, respiratory distress or unusual pallor were encountered. Two premature infants who received 100 mg./Kg. in four divided doses per day for six days had blood drawn on the fifth day. There was no evidence of abnormal accumulation of erythromycin in the blood. Both premature infants had a serum level of 5 µg./ml.

The aqueous suspension was well received by the older children. They liked the flavor of the medication and did not report a bitter taste. None refused the medication. No skin rashes, vomiting, gastrointestinal upset, or toxic reactions were encountered. Infants took the suspension without any untoward reactions.

COMMENTS

The optimum dosage of propionyl erythromycin lauryl sulfate would appear to be about 5 mg./lb. of body weight every six hours. The data of Griffith and the irregularity of smaller dosages in producing uniformly high serum levels would tend to support this. The small series studied showed that with this dosage fewer

failures of treatment were encountered. It must be remembered that the number of children in this series is small and that any conclusion drawn from these results must be considered with caution. The newborn and premature infants did not show any evidence of accumulation or toxic reactions to propionyl erythromycin lauryl sulfate in the large dosages given. While it is possible that some infants may show an idiosyncrasy to this antibiotic, the data presented would appear to indicate that several times the therapeutic dosage may be given to infants without harmful effect. The specific lack of bitter taste in the aqueous suspension and the failure of gastric juice to inactivate propionyl erythromycin lauryl sulfate make this form of erythromycin more acceptable to children; also, high blood levels of the antibiotic are provided.

CONCLUSION

Propionyl erythromycin lauryl sulfate gives serum levels in children that are 2.5 times greater than those reported for propionyl erythromycin ester in adults when a dosage of 5 mg./lb. every six hours is given orally in the fasting or nonfasting state. The aqueous suspension of this antibiotic was well tolerated by children and no untoward effects were seen. Large dosages had no effect on premature or newborn infants. No accumulation of the antibiotic occurred in the serum of 2 premature infants. A satisfactory clinical response was seen in children treated with propionyl erythromycin lauryl sulfate.

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Experiences with Triacetyloleandomycin in a Rheumatic Fever Prophylaxis Clinic

THOMAS W. MOU

*Department of Preventive Medicine, State University of New York,
Upstate Medical Center at Syracuse, Syracuse, N. Y.*

The effectiveness of various prophylactic agents in preventing recurrences of acute rheumatic fever by diminishing the risk of acquiring new group A streptococcal infections has been demonstrated in both military and civilian studies.¹⁻⁵ Sulfonamides and penicillin are highly satisfactory drugs for this purpose but allergic reactions to them are common. Glazer⁶ states that 2 to 2.5 per cent of children, 5 per cent of non-allergic adults, and 15 per cent of allergic subjects may have reactions to parenterally administered penicillin. Dowling and Lepper⁷ noted dermatitis, fever, and/or conjunctivitis in 6.2 per cent of patients receiving sulfathiazole, 2.4 per cent of those receiving sulfapyridine, and 2 per cent of those receiving sulfadiazine.

The search for effective chemotherapeutic and antibiotic agents that may be used for patients allergic to the sulfonamides and/or penicillin has been a continuing one in our rheumatic fever prophylaxis clinic. Since laboratory data and early clinical reports⁸⁻¹⁰ indicated that group A streptococci were very sensitive to triacetyloleandomycin,* the present study was undertaken to determine the usefulness and suitability of the drug in a long-term rheumatic fever prophylaxis program.

METHOD OF STUDY

The subjects were 55 patients in the rheumatic fever prophylaxis clinic. All had been maintained previously on oral benzathine penicillin for periods ranging from three months to three years. These patients had been admitted to the clinic only if they had rheumatic heart disease or if the staff had accepted the diagnosis of previous rheumatic fever. The youngest patient was 5 years old, the oldest 69 years, and the mean age for all patients was 31 years. One-third, or 18, were men and two thirds (37) were women.

Using the double blind method, the patients were divided into two groups: one continued with a single daily oral dose of 200,000 units of benzathine penicillin,† while the other received one 250 mg. triacetyloleandomycin capsule daily. After four months, the treatment schedules of the two groups were reversed for a similar four month period. The change in treatment was made during February, usually the peak month for streptococcal infections in this community. This assured for each patient, essentially similar amounts of experience with each form of medication both in months of higher and lower streptococcal prevalence. The patients were not informed of any change in medication nor that they were receiving an antibiotic other than penicillin.

This study was supported by grants from Wyeth Laboratories, Inc., and F. K. Kilian.

* The trade name of Wyeth Laboratories for triacetyloleandomycin is Cyclamycin.

† The trade name of Wyeth Laboratories for benzathine penicillin is Bicillin.

The patients were seen at monthly intervals at which time they were provided with medication for the following month. Before this, however, they were questioned as to whether acute respiratory illnesses or sore throat had occurred in them or their families during the intervening period. Inquiry also was made about rash, epistaxis, or joint manifestations. They were then interrogated intensively about the regularity with which they had taken the prescribed medication and whether they had noted reactions, such as rash or other skin lesions, and gastrointestinal disturbances, such as nausea, vomiting, excessive flatulence, or diarrhea. The interviewing physician was unaware of the treatment which the patient was receiving.

Throat cultures and blood specimens* for erythrocyte sedimentation rate, hematocrit, C-reactive protein, and antistreptolysin-O determinations were obtained at each visit.

RESULTS

The 55 patients accumulated 197 patient-months experience with benzathine penicillin (200,000 u. daily) and 181 patient-months with triacetyloleandomycin (250 mg. daily). Twenty-four (44 per cent) of the patients took their medication on schedule throughout the eight month period of observation and 35 (64 per cent) completed six or more months of the prophylactic treatment. Fifteen patients (27 per cent) had five or fewer months of antibiotic treatment; 5 patients (9 per cent) required changes in their medication because of toxic reactions.

The monthly throat cultures for streptococci were consistently negative in both groups. There were three instances of antistreptolysin-O titer increases of 20 per cent or more (considered to be a significant increase in this laboratory); each was related to failure to take the medication. One of these, a patient who had failed to take her prescribed triacetyloleandomycin for two months, was found to have a tripling of her antistreptolysin-O titer. A second patient failed to take her triacetyloleandomycin for one week; subsequently, her titer almost doubled. The third patient omitted her benzathine penicillin for one month and her titer increased by half as much again.

Five patients (9 per cent) of the 55 that received the triacetyloleandomycin had toxic reactions. These included rectal burning in 2 patients, one after two and a half months of daily ingestion of the drug, and the other after one month. Gastrointestinal symptoms (diarrhea, flatulence, and nausea) occurred on two separate occasions in a third patient. A fourth developed severe headache following several days of treatment and again during a second course. The fifth noted a fine, erythematous rash on her trunk ("like prickly heat") one week after she started on the triacetyloleandomycin; the rash persisted for the month that she took the medication but disappeared after it had been discontinued for a week. (This patient had

* Throat swabs were plated directly on sheeps' blood agar and again after incubation in modified Pike's broth.¹¹ Sedimentation rates and hematocrits were measured in Wintrobe tubes by a standardized technique. C-reactive protein determinations were made with either Schieffelin or Difco antisera. Antistreptolysin-O was titrated by a method in use in this laboratory.¹²

had hives when treated previously with penicillin and possibly streptomycin, for subacute bacterial endocarditis.) The remaining 50 patients had no untoward effects during their periods of observation.

DISCUSSION

Group A beta-hemolytic streptococci are not only very sensitive to penicillin but appear to be incapable of acquiring significant resistance to this drug. The available data¹⁰ suggest that these organisms are similarly sensitive to the action of triacetyloleandomycin.

Throat cultures were consistently negative for streptococci in both groups, although the occurrence of group A beta-hemolytic streptococcal infections in the community at large was relatively high during the 1958–1959 season. In addition, several of the patients included in this study are participating in a respiratory disease family study in which biweekly throat cultures have indicated a high prevalence of streptococcal infections in family members other than those receiving the prophylaxis. While blood levels of triacetyloleandomycin were not determined, Shubin et al¹³ reported blood serum levels of 0.5 to 1.0 µg./ml. following the ingestion of a single 250 mg. capsule. This level is probably sufficient to suppress the growth of group A streptococci.

The almost total lack of significant antistreptolysin-O titer increases among the 55 patients suggests that subclinical infections were uncommon. Although we do not have a control group of patients who did not receive prophylactic medication, earlier data from this clinic¹⁴ show a steady decline in the median antistreptolysin-O value for the entire clinic population, indicating that prophylactic measures not only prevent clinical disease but reduce (or minimize) subclinical infections as well. This is consistent with the findings of Miller et al¹⁵ that rheumatic children on prophylaxis have fewer streptococcal infections than their unprotected siblings.

Toxic reactions to triacetyloleandomycin were found to be relatively infrequent, mild in nature, and of short duration following discontinuance of the drug. This is similar to the experience reported by Albright and Hall.¹⁶ Three of the five toxic reactions were related to the gastrointestinal tract and were more annoying than serious. It is difficult to interpret headache as a toxic reaction, although it occurred on two separate occasions in the same patient. The development of a skin rash in a patient who supposedly had had an allergic reaction during previous intensive antibiotic therapy for subacute bacterial endocarditis is of interest but not necessarily related.

SUMMARY

Triacetyloleandomycin was used in a rheumatic fever prophylaxis clinic where it appeared to suppress clinical and subclinical streptococcal infections in a manner similar to penicillin. The daily ingestion of the drug was well tolerated for a four month period by almost all patients. Toxic reactions were infrequent and mild.

While penicillin remains the drug of choice for oral streptococcal prophylaxis, triacetyloleandomycin appears to be worthy of further trial, especially in those patients whose hypersensitivity to penicillin precludes the latter's use.

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Treatment of Severe Staphylococcal Infections in Infancy and Childhood with Vancomycin

HARRIS D. RILEY, JR. AND NELL J. RYAN

*Department of Pediatrics and the Children's Memorial Hospital,
University of Oklahoma Medical Center, Oklahoma City, Okla.*

Vancomycin,* a new antibiotic isolated from *Streptomyces orientalis*, has bactericidal activity and is particularly effective against gram-positive bacteria. It is of particular interest that staphylococci develop very little resistance after several subcultures in the presence of the drug and no cross resistance with other antibiotics has been observed. It has particular usefulness in the treatment of staphylococcal infections, especially those due to organisms resistant to other antimicrobial agents.

The frequency and importance of staphylococcal infections in infants and children is well recognized. The present report includes clinical and laboratory information concerning the treatment of a group of infants and children with severe staphylococcal infections with this drug.

MATERIALS AND METHODS

Twenty-five infants and children in the Infectious Disease Division of the Children's Memorial Hospital with severe staphylococcal infections were treated with vancomycin. The age of the patients ranged from 2 weeks to 15 years. Included in the group are 9 patients with septicemia, 6 with pneumonia, 2 with bacterial endocarditis, 2 with osteomyelitis, 4 with extensive cellulitis and skin abscesses, and 2 patients with severe periorbital cellulitis. All patients were considered to have severe staphylococcal infections and many had been treated unsuccessfully with other antimicrobial agents prior to the initiation of therapy with vancomycin.

Appropriate cultures were obtained before, during, and after therapy in most cases. Susceptibility studies of staphylococcal isolates were carried out by the tube dilution technique. All patients were followed with complete hemograms, urinalyses, and blood urea nitrogen studies at regular intervals. Liver function studies were carried out in some of the patients. All patients were carefully followed from a clinical standpoint for the development of untoward effects to the drug. Careful attention was devoted to the development of auditory impairment.

The drug was given intravenously in all cases. Various techniques of administration were employed. After mixing with distilled water the drug was injected into the tubing of a constant intravenous infusion requiring four to five minutes to give each 10 ml. of the solution and in other cases was added to a constant intravenous infusion so that the desired amount was given over a period of 20 to 30 minutes every 8 to 12 hours. In other cases it was added directly to the intravenous infusion and allowed to drip slowly over a 24 hour period until the estimated daily dose was received. In certain other cases where long-term therapy was desirable, it was administered by means of a constant infusion through a venous cut-down for

* The trade name of Eli Lilly & Co. for vancomycin is Vancocin.

several consecutive days. The dosage ranged from 10 to 68 mg./lb./day, the total daily dose being divided into two or three aliquots. The duration of treatment varied with the individual case. The longest continuous therapeutic trials were 15 and 16 days respectively. Intermittent courses of therapy were administered to several patients.

RESULTS AND DISCUSSION

All patients were considered to have serious staphylococcal disease that commonly was hospital acquired. In many instances the patient had a serious or generally fatal underlying disorder (table I). Many of the patients had been previously treated with various antibiotics, usually without success, prior to the initiation of vancomycin therapy. Other details of the patient population and management including clinical manifestations, dose, duration of therapy, results, and untoward effects are given in table I.

Vancomycin found its greatest usefulness as initial therapy in patients with serious life-threatening staphylococcal infections. The fact that the agent must be administered intravenously proved to be a significant obstacle in the long-term treatment of many patients, particularly infants and small children. Even when phlebitis at the site of injection did not give rise to fever and other constitutional manifestations, the local effect on the venous channels often precluded prolonged administration. In many such instances the "course" of antimicrobial therapy was completed with another agent or a combination of agents which could be administered orally or intramuscularly.

Septicemia. Of the 9 patients with septicemia a satisfactory response was obtained in 8 (table I). Three patients had acute leukemia with a complicating staphylococcal bacteremia. Although clinical improvement was difficult to evaluate because of the primary disease, blood cultures in all 3 patients promptly became sterile following therapy with vancomycin. Cases 1, 2, 4, and 6 had been extensively treated with erythromycin, chloramphenicol, and other antibiotics without eradication of the infection. Vancomycin resulted in a prompt clinical and bacteriological response in each instance. One patient (case 1) had been under treatment for 18 days with erythromycin and, although there was some clinical improvement, blood cultures continued to yield coagulase-positive staphylococci. On vancomycin therapy the blood cultures became sterile in 48 hours and remained so after 12 days of therapy. A 17 day old infant (case 9) was admitted with staphylococcal septicemia, pneumonia, meningitis, extensive skin abscesses, and, at autopsy, multiple hepatic abscesses. The patient was moribund on admission and died 15 hours later. She received only two doses (total of 500 mg.) of vancomycin in the interval; blood cultures were still positive at autopsy.

In view of the high mortality associated with this form of staphylococcal disease, the results achieved in this group of patients appear to be significant. Although 2 patients died, 1 (case 5) died of her underlying disease and not the complicating infection (in fact, blood cultures at autopsy were sterile) and the other (case 9) expired of overwhelming generalized infection before any benefits of therapy could be realized.

Bacterial Endocarditis. Two children with staphylococcal bacterial endocarditis

TABLE I
Pertinent Details and Clinical Results of 25 Patients with Staphylococcal Infections
Treated with Vancomycin

Pt.	Age	Clinical problem	Dose, mg./lb./day	Duration, days	Result	Side effect
<i>Septicemia</i>						
1	15 yr.	Furunculosis and septicemia	16.7	12	Prompt clinical and bacteriological improvement. Previously treated with erythromycin for 18 days without effect	None
2	15 yr.	Acute leukemia with staphylococcal parotitis and septicemia	16.6	16	Blood cultures became sterile and parotitis improved but clinical improvement difficult to evaluate because of basic disease. Previously treated with multiple antibiotics unsuccessfully	None
3	5½ yr.	Acute leukemia with septicemia	20.0	4	Poor clinical response but blood cultures became sterile in 2 days. Therapy terminated because of severe phlebitis at site of injection	Severe phlebitis
4	9 yr.	Septicemia and purulent arthritis of hip	40.0	15	Prompt clinical improvement. Blood cultures became negative after 72 hours. Previously treated without success with multiple drugs	None
5	4 yr.	Acute leukemia and septicemia	19.0	3	Patient died on third day of therapy of intracranial hemorrhage. Blood culture at autopsy sterile	None
6	6 mo.	Pyoderma and septicemia	20.0	20	Unsuccessfully treated with chloramphenicol and erythromycin. Blood cultures became sterile in 48 hours on vancomycin with marked clinical improvement	None
7	7 yr.	Severe malnutrition with skin abscesses and septicemia	45.0	5	Prompt response	None
8	12 yr.	Extensive furunculosis, skin abscesses and septicemia	15.0	5	Prompt clearing of skin lesions and blood cultures became sterile	Mild phlebitis
9	17 days	Staphylococcal septicemia, meningitis, pneumonia, cellulitis, and hepatic abscesses	30.0	½	Patient moribund on admission and died 15 hours later. Cultures of blood and liver abscess at autopsy revealed <i>M. pyogenes</i>	None

Table I Continued on Page 911

TABLE I (Continued)

Pertinent Details and Clinical Results of 25 Patients with Staphylococcal Infections Treated with Vancomycin

Pt.	Age	Clinical problem	Dose, mg./lb./day	Duration, days	Result	Side effect
<i>Bacterial Endocarditis</i>						
10	10 yr.	Rheumatic heart disease with staphylococcal endocarditis	26.0	5	Had recurrent attacks of chills and fever for 5 months. Critically ill with evidence of central nervous system embolization on admission. Prompt drop in fever and blood cultures became sterile in 48 hours. Patient died of massive cerebral embolus on 5th day of therapy	None
11	6 yr.	Staphylococcal endocarditis with secondary osteomyelitis of left femur and os calcis	20.0	5	No significant response in fever. Some clinical improvement and blood cultures became sterile. Persistent fever possibly due to drug	Febrile reaction and phlebitis
			30.0	5		
<i>Pneumonia</i>						
12	4½ yr.	Staphylococcal pneumonia and pneumothorax following open cardiomy	27.0	4	Prompt clinical response with drop in temperature to normal. Subsequent febrile spike probably due to chemical phlebitis	Phlebitis and fever
13	14 days	Severe pneumonia and empyema	48.0	9	Patient moribund on admission. Prompt clinical and radiological improvement on vancomycin and pleural drainage. Cultures promptly reverted to sterile	None
14	13 yr.	Pneumonia. One blood culture yielded <i>Staph. albus</i>	4.3	5	Initial clinical response but pneumonia showed radiological progression followed by recurrence of fever. Prompt response to chloramphenicol. Etiology of pneumonia ? and dose small by error	None
			8.6	3		
15	5 yr.	Pneumonia, bilateral	30.0	6	Prompt clinical and bacteriological response	None
16	6½ mo.	Pulmonary nocardiosis with secondary staphylococcal pneumonia and septicemia	24.0	6	Little clinical response, but blood cultures became sterile in 48 hours. At autopsy extensive nocardiosis of lung but cultures negative for <i>M. pyogenes</i>	None

Table I Continued on Page 912

TABLE I (Continued)

Pertinent Details and Clinical Results of 25 Patients with Staphylococcal Infections Treated with Vancomycin

Pt.	Age	Clinical problem	Dose, mg./lb./day	Duration, days	Result	Side effect
17	1½ mo.	Pneumonia and empyema with multiple lung abscesses and pulmonary collapse	68.0 57.5 20.0	3 7 13	Severe pneumonia and empyema treated extensively with multiple antibiotics without improvement. Developed multiple lung abscess and pulmonary collapse. Prompt response to vancomycin and lungs roentgenographically normal at discharge	Transitory auditory impairment. Hearing normal 3 mo. after therapy
<i>Osteomyelitis</i>						
18	5 yr.	Osteomyelitis, right femur	25.0	12	Prompt clinical response. Temperature returned to normal in 48 hours, and cultures reverted to negative	Phlebitis and rash
19	1½ yr.	Osteomyelitis, right humerus	30.0	13	Prompt clinical response with drop to normal of temperature. Satisfactory bacteriological response	None
<i>Soft-Tissue Infections</i>						
20	2½ yr.	Periorbital cellulitis, severe; mixed infection (staphylococcal and streptococcal)	23.0	3	Satisfactory clinical response. <i>M. pyogenes</i> eradicated in 24 hours, but beta-hemolytic <i>Streptococcus</i> persisted for 72 hours	Mild phlebitis
21	15 yr.	Periorbital cellulitis, severe; ? sinus thrombosis; extensive cellulitis of face	20.0	5	Satisfactory clinical and bacteriological response	Possible drug fever
22	3 mo.	Ritter's disease (dermatitis exfoliativa neonatorum) with extensive secondary staphylococcal pyoderma and skin abscesses	28.0	4	Patient died on 4th day of therapy. However, skin cultures negative after 36 hours of therapy and death felt to be due to Ritter's disease	None
23	10 yr.	Extensive subcutaneous abscess of thigh	30.0	6	Temperature promptly returned to normal and abscess cleared by 3rd day of therapy	None
24	15 yr.	Staphylococcal abscess of gluteal region	10.0	3	Some clinical improvement but necessary to discontinue drug after 2 days because of febrile spike and chill after each injection	Drug fever
25	6 mo.	Multiple skin abscesses; congenital heart disease	20.0	8	Prompt clinical and bacteriological response	None

were treated with vancomycin. A 10 year old boy with rheumatic heart disease (case 10) had experienced recurrent attacks of chills and fever over a period of five months. On admission he was critically ill with evidence of embolic phenomena of the central nervous system. On vancomycin, there was a prompt drop to normal of temperature and blood cultures were sterile in 48 hours. However, the patient died of a massive cerebral embolus on the fifth day of therapy.

The other patient with bacterial endocarditis had undergone extensive therapy prior to admission with other antimicrobial agents and adrenal steroids without improvement. She was started on vancomycin in a dose of 20 mg./lb./day; however, although there was clinically some improvement, fever persisted (fig. 1). Despite the persistence of fever, blood cultures were sterile 34 hours after initiation of vancomycin therapy. On discontinuing vancomycin, the temperature promptly returned to normal. In all probability the febrile response was due to the drug as marked chemical phlebitis developed at the site of each injection. The patient's therapy was completed with kanamycin.

Geraci et al¹ reported good results with relatively short courses of vancomycin in micrococcal endocarditis in older patients.

Pneumonia. In 5 of 6 cases of pneumonia treated with vancomycin there was unequivocal cultural evidence of a staphylococcal etiology. The results were good in these 5 patients. The 1 patient with pneumonia that did not respond satisfactorily to vancomycin was a 13 year old girl (case 14) in whom the etiology of the pneumonia was uncertain. Only one blood culture yielded *Staphylococcus albus*. By error the dose of vancomycin given was only 4.3 mg./lb./day. There was an initial clinical response but radiologically there was progression of the pneumonia followed by the development of pleural effusion. The dose of vancomycin was doubled for an additional three days without significant improvement. The response to chloramphenicol was prompt.

Another patient, case 17, is particularly noteworthy. This 6 week old boy was seriously ill on admission with extensive staphylococcal pneumonia and empyema. He had been vigorously treated with erythromycin and chloramphenicol before admission. The staphylococcal strain was sensitive in vitro to kanamycin and initially there was a satisfactory clinical and bacteriological response to this drug; however,

FIG. 1. Drug fever due to vancomycin in a case of bacterial endocarditis. Although temperature remained elevated for eight days during vancomycin therapy, the patient exhibited clinical improvement and blood cultures were sterile 34 hours after initiation of treatment.

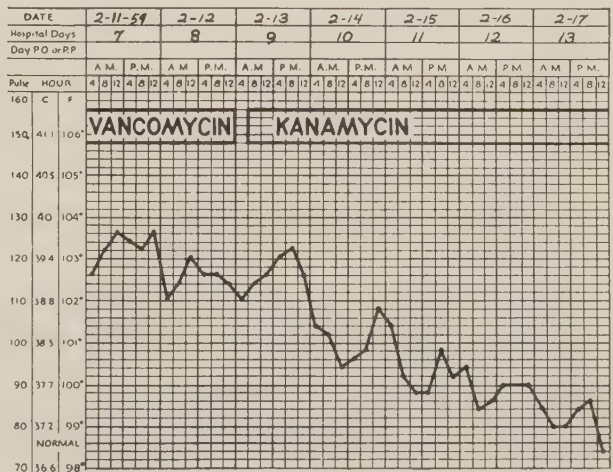


TABLE II
In Vitro Activity of Vancomycin Against Strains of *Staphylococcus aureus*

Total no. of strains	Inhibitory concentration, $\mu\text{g.}/\text{ml.}$						
	10.0	6.25	5.0	3.1	2.5	1.56	1 or less
24	0	0	4	2	11	2	5

cultures of blood and spinal fluid again became positive for staphylococci on this therapy. In the interval he had developed multiple lung abscesses and pulmonary collapse, with deterioration of his clinical condition. On vancomycin, all cultures became negative and remained so and there was immediate improvement in his clinical condition. On discharge, the lung fields were roentgenographically normal.

Several other patients with staphylococcal pneumonia who were considered gravely ill made satisfactory and in some cases dramatic responses to treatment with vancomycin.

The grave prognosis in infants and children with this manifestation of staphylococcal infection has been well documented.^{2,3}

Osteomyelitis. Two children with staphylococcal osteomyelitis were treated for 12 and 13 days respectively with vancomycin with a prompt clinical and bacteriological response in both instances. The treatment program in both patients was completed with the use of oral antimicrobial agents. Another patient with osteomyelitis secondary to a staphylococcal septicemia obtained a satisfactory bacteriological response to vancomycin but clinical response was more difficult to evaluate because of probable drug fever due to vancomycin.

Soft-Tissue Infections. All 6 patients had severe life-threatening soft-tissue infections. Two children with extensive periorbital cellulitis responded satisfactorily to vancomycin. In 1 patient (case 20) there was prompt eradication of the staphylococci but beta-hemolytic streptococci persisted for 72 hours although clinically the inflammatory response had cleared.

One infant with Ritter's disease and secondary staphylococcal skin abscesses died; however, cultures of the skin had become negative and the cause of death was felt to be the primary disorder.

Drug Susceptibility Tests. The results of susceptibility tests of representative strains of *Staph. aureus* are shown in table II. Of the 24 strains tested, all were inhibited by concentrations of 5.0 $\mu\text{g.}/\text{ml.}$ or less. Only 6 strains required a concentration greater than 2.5 $\mu\text{g.}/\text{ml.}$ for inhibition. Virtually all of the strains were resistant to penicillin and tetracycline, and many to erythromycin and chloramphenicol.

In Vitro. In vitro resistance to vancomycin during the course of therapy did not occur in any case.

Side Effects. Four types of untoward reactions were noted. These were local irritation of the tissues, principally a chemical phlebitis, drug fever, skin rash, and auditory impairment. Five patients in the series developed phlebitis of some degree at the site of injection. It was not possible in all cases to tell whether this was due to leakage of the antibiotic solution out of the vein during administration but certainly this occurred in some cases resulting in an inflamed, painful soft-tissue swell-

ing at the site of injection. This usually subsided in a few days without residual. In 2 instances the phlebitis was severe enough to necessitate termination of therapy with vancomycin. In the other patients it was milder and, although it often caused some constitutional reaction, did not force termination of treatment. Both of the patients with severe phlebitis had an associated temperature elevation which interfered with evaluation of the therapeutic response.

Drug fever probably occurred in 3 patients. In 2 instances this was associated with severe phlebitis and both patients were quite ill following each injection. One patient had a marked febrile spike and chill following each injection of vancomycin.

The untoward reactions of phlebitis and drug fever occasionally represented a significant problem in the evaluation of response of the patient. In 1 child with bacterial endocarditis the temperature remained elevated for eight days during vancomycin therapy; however, during this period the patient showed continued clinical improvement and blood cultures were sterile within 34 hours after therapy was initiated (fig. 1). During the period before the results of blood cultures were available, it was difficult to determine whether vancomycin was therapeutically effective in view of the continued temperature elevation.

One patient developed a rash, which was felt to be due to vancomycin. This was an erythematous urticarial-like rash which cleared promptly when vancomycin was discontinued; with some of the earlier lots skin flushing immediately after injection occurred.

One infant with severe staphylococcal pneumonia, empyema, and pulmonary collapse who was in a critical state developed auditory impairment following vancomycin; however, this patient had previously been treated with kanamycin. He received very large doses of vancomycin (68 mg./lb./day for three days, 57.5 mg./lb./day for seven days and 20 mg./lb./day for 13 days). Vancomycin therapy was accompanied by dramatic improvement and its use was felt to be lifesaving. Three months after vancomycin therapy hearing was entirely normal. Repeated audiometric testing since that time has revealed no hearing impairment.

Most of the cases of phlebitis and drug fever in our experience occurred with the earlier lots of vancomycin. With improvement in manufacture, these reactions became less frequent and severe. One patient who exhibited a febrile response to vancomycin was given a repeat course with no evidence of untoward reaction.

Blood urea nitrogen and creatinine determinations, urinalyses, and liver function tests were performed at regular intervals. There was no evidence of renal or hepatic toxicity in the series of patients under treatment. There was likewise no evidence of hematopoietic disturbance.

In no case was there any evidence of the development of superinfection. Particular precautions were observed to detect the occurrence of gram-negative organisms as a consequence of vancomycin therapy but this did not occur.

Several techniques of intravenous administration of this agent were utilized as described earlier. No conclusive opinion was developed as to the most feasible technical method of administration. However, in young infants we have the clinical impression that intermittent injection into the tubing of a constant intravenous infusion requiring four to five minutes to give each 10 ml. of the solution resulted in less venous sclerosis than did other methods. Rotation of veins for infusions is also helpful in reducing this problem.

COMMENTS

In this series of patients with serious staphylococcal infections often superimposed on a severe life-threatening primary disease, the therapeutic results with vancomycin are impressive. While certain bacteriostatic antimicrobial agents used singly or in combination may be adequate in the management of mild staphylococcal infections, they are all too often ineffective in severe infections. In addition, many of the other antistaphylococcal agents, while effective, have significant toxicity potential. Vancomycin appears to be an extremely useful agent in the treatment of severe staphylococcal infections. It is bactericidal in action and inhibits staphylococci in lower concentrations than most other antimicrobial agents. Effective serum concentrations are readily attained and there is no cross resistance with other antibiotics. The major disadvantage of vancomycin therapy is the fact that it must be administered intravenously. Although many patients with severe staphylococcal infections may already be receiving intravenous infusions, the necessity of intravenous administration is still an obstacle particularly in infants and young children if prolonged therapy is required. While the untoward effects of vancomycin are not uncommon, they are generally not serious in contrast to those of many other specific antistaphylococcal agents. Many of the side effects associated with early lots of vancomycin have been eliminated completely or in part by the more refined forms now available.

Vancomycin therapy should find its most useful place as initial therapy in patients with serious staphylococcal disease. After eradication of the infection the "course" of therapy may be continued with other antimicrobial agents which are easier to administer.

SUMMARY

Vancomycin was employed therapeutically in the management of 25 infants and children hospitalized with serious staphylococcal infections. In view of the severity of the infections in this group of patients and the nature of their underlying illnesses, the therapeutic effects are considered significant. All strains of *Staph. aureus* were inhibited by vancomycin in concentrations of 5 µg./ml. or less and the majority required less than 2.5 µg./ml. The majority of the strains were resistant to other commonly employed antimicrobial agents. While untoward mild side effects from the drug were relatively common, particularly with earlier lots, the side effects were not serious and therapy had to be terminated in only 2 cases because of side reactions.

Vancomycin is felt to be a valuable agent in the treatment of serious staphylococcal disease.

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Clinical Evaluation of Ristocetin in Children

RICHARD KOCH, CHARLES P. DRIES, AND LYAL D. ASAY

*Department of Pediatrics of the University of Southern California School of Medicine
and the Childrens Hospital of Los Angeles, Los Angeles, Calif.*

Last year at this Symposium we presented data to show that ristocetin* must be given in a dosage ranging from 37 to 50 mg./Kg. of body weight per day and administered every 8 to 12 hours, and that on rare occasions dosages as high as 75 mg./Kg. of body weight per day were necessary to eliminate particularly resistant infections in children.

It was also shown that this antibiotic could also be given intramuscularly to small children and infants, providing it was combined with 1 mg. of hydrocortisone per 100 mg. of ristocetin administered.

During the past year, these studies have been extended and a total of 90 hospitalized patients ranging in age from 10 days to 15 years have been treated with intramuscular and intravenous ristocetin.

The method of administration varied, depending upon the age of the child, accessibility of veins, and the seriousness of his condition, i.e., the younger the infant and the less serious the disease, the greater the likelihood of using ristocetin intramuscularly. If the child was older or critically ill, ristocetin was administered intravenously. Three dosage schedules were compared: 25, 50, and 75 mg./Kg. of body weight per day. Usually the antibiotic was administered in equally divided doses every eight hours. When the intramuscular route was employed, 1 mg. of hydrocortisone was added to each 100 mg. of ristocetin administered. The nature of the disease, organism isolated, clinical response, and toxicity were recorded.

RESULTS

A total of 90 patients were treated. Sixty of these received ristocetin alone and 30 received additional antibiotics because they were so seriously ill that it was considered unwise to rely upon a single drug. Fifty-seven (63 per cent) of the patients had staphylococcal infections and 37 per cent had a variety of other organisms.

As can be seen in table I, ristocetin proved to be most effective in treating infections in which there were no membranes to cross or abscesses to penetrate. The poorest results were found in meningitis, pneumonia with empyema, and in endocarditis in which one third were classified as treatment failures. It was particularly efficacious in patients with sepsis, for all but 1 of 9 recovered, and in soft-tissue infections, such as cellulitis and pneumonia without empyema, in which all 50 patients recovered. One infant with sepsis died shortly after admission to the hospital.

Table II is quite illuminating, for it demonstrates the necessity of employing adequate dosage for therapy. Twenty-three per cent of the patients receiving a

* The trade name of Abbott Laboratories for ristocetin is Spontin.

TABLE I
Clinical Results of Ristocetin Therapy

Diagnosis	Organism	Total no.	Cured	Unimproved	Died
Pneumonia with empyema } Sepsis	<i>Staphylococcus</i>	15	11	4	—
	<i>Pneumococcus</i>	1	—	1	—
	<i>Staphylococcus</i>	10	8	—	1
Endocarditis	<i>Enterococcus</i>	—	1	—	—
	<i>Staphylococcus</i>	6	4	2	—
Meningitis	<i>Staphylococcus</i>	2	—	2	—
Pericarditis	<i>Staphylococcus</i>	1	—	—	1
Cellulitis	<i>Staphylococcus</i>	16	15	1	—
Osteomyelitis	<i>H. influenzae</i>	2	1	—	—
	Unknown	—	—	1	—
Enterocolitis	<i>Staphylococcus</i>	1	1	—	—
Respiratory tract infections	Various	36	35	—	1*
Total		90	76	11	3

* This patient also had fibrocystic disease of the pancreas.

divided dose of 25 mg./Kg. failed to respond, whereas only 10 per cent of those receiving 50 mg./Kg. failed to respond. Four patients who did not respond to a lower dosage responded to 75 mg./Kg./day. One patient receiving 75 mg./Kg./day was a 7½ year old boy who developed fulminating pericarditis and septicemia following surgical repair of an atrial septal defect. Ristocetin, erythromycin, and chloramphenicol were utilized but failed to prevent this child's death.

TOXICITY

Only one of these patients demonstrated thrombocytopenia, which has been reported elsewhere.² As recorded in table III, 68 of the 90 patients had no toxic reactions. The most common abnormality was eosinophilia noted in 11 patients. Six patients developed neutropenia and 4 patients demonstrated macular erythematous rashes. All such manifestations of toxicity disappeared in less than one week after discontinuance of medication. Local reactions to injection were tenderness, erythema, and induration in 6 patients receiving intramuscular injections and thrombophlebitis in 4 patients receiving ristocetin intravenously. One patient developed a sterile abscess and many of the older children complained of pain when ristocetin was given intramuscularly. Local reactions to ristocetin were surprisingly few, al-

TABLE II
Failure to Respond to Ristocetin Related to Dosage

Dosage, mg./Kg./day	Number patients	Number of failures	Percentage of failures
25	35	8	23
50	50	5	10
75	5	1	20

TABLE III

Toxicity Noted in 90 Patients Treated with Ristocetin

Toxic manifestation	Number of patients
Eosinophilia	11
Neutropenia	6
Rash	4
Thrombophlebitis	4
Tenderness, erythema, and induration	6
Sterile abscess	1
Thrombocytopenia	1

though 75 out of 82 patients received treatment for from 2 to 16 days without difficulty. Table IV shows the relationship between dosage and incidence of side reactions. As noted by Hertig et al,⁴ toxicity appeared to be directly proportional to the dosage used.

DISCUSSION

Ristocetin is an extremely useful antibiotic and when administered carefully is quite safe. Its greatest usefulness is in treating infections caused by gram-positive organisms, particularly the troublesome hemolytic coagulase-positive staphylococci.^{4,7} Toxic reactions, while rather frequent, are usually minor, seem to be related to dosage (table IV), and appear to be reversible.

It has usually been given intravenously because of severe tissue reaction to intramuscular administration. Incorporating 1 mg. of hydrocortisone with each 100 mg. of ristocetin minimizes this problem in infants and small children and extends its usefulness to this group of patients in whom intravenous administration of antibiotics is technically difficult.

Ristocetin does not enter into the cerebrospinal fluid across the uninflamed meninges. Apparently it also fails to cross in sufficient concentration to be effective when the patient has meningitis, as evidenced by the 2 treatment failures recorded in our series, although Kanner⁸ reported a cure in a 32 year old woman with meningitis.

One case was particularly interesting and has been outlined graphically in figure 1. This child with staphylococcal meningitis responded well to chloramphenicol, 100 mg./Kg./24 hours, and ristocetin, 25 mg./Kg./24 hours, with prompt clearing

TABLE IV

Toxicity Related to Dosage in 90 Patients Treated with Ristocetin

Dosage, mg./Kg./day	Number of patients	Number of reactions	Percentage of reactions
25	35	5	17
50	50	13	26
75	5	3	60

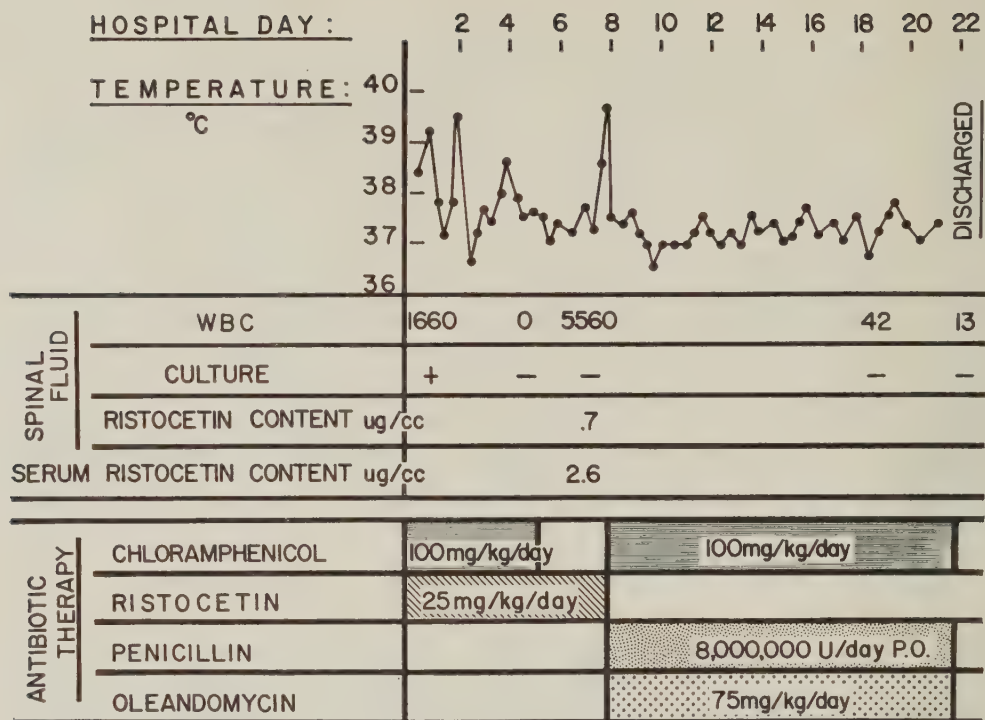


FIG. 1. The clinical course of a 5 year old boy with staphylococcal meningitis, showing relapse while on ristocetin therapy alone.

of his spinal fluid; however, when chloramphenicol was discontinued, he relapsed. When chloramphenicol was given again and ristocetin stopped, he again responded to therapy. The cerebrospinal fluid content of ristocetin at the time of relapse was less than 0.7 $\mu\text{g./ml.}$, compared to a serum level of only 2.6 $\mu\text{g./ml.}$ These results suggest that ristocetin administered in this dose is not adequate for efficacious therapy.

Whenever ristocetin is utilized for therapy, certain precautions are necessary. A routine blood count should be performed three times weekly⁹ and a blood urea nitrogen determined prior to or soon after therapy is begun. Dosage should be reduced accordingly in the presence of renal failure, which may be encountered when treating severe endocarditis. Serious hematologic changes, such as neutropenia or thrombocytopenia, tend to occur after prolonged therapy with high dosage¹⁰ or in the presence of renal failure.² The latter results in elevated serum levels of ristocetin due to failure of renal excretion of the antibiotic in the urine, resulting in greater toxicity. The exact status of ristocetin in relationship to other antibiotics remains to be clarified. Only Rantz and Jawetz¹¹ have reported significant treatment failures in treating staphylococcal infections;¹⁰ however, the dosage administered in 2 of their cases was low (approximately 25 to 37 mg./Kg./day, estimated). Two of these patients also received vancomycin without benefit.

It is our clinical impression that both ristocetin and vancomycin are valuable antibiotics and will provide the clinician with gratifying results if used wisely and judiciously.

SUMMARY

1. Ristocetin is a valuable antibiotic for treating serious infections caused by staphylococci.
2. Recommended dosage is 50 mg./Kg./24 hours, given in divided doses every 8 to 12 hours.
3. It may be administered intramuscularly to infants and small children if one mg. of hydrocortisone is incorporated with each 100 mg. of ristocetin.
4. Toxic reactions are relatively mild and reversible, consisting of eosinophilia, thrombocytopenia, rash, thrombophlebitis, and local myositis. Serious reactions such as neutropenia usually result from large doses administered over a period of time.
5. Ristocetin is not very effective for treating meningitis, presumably because its large molecular size prevents adequate penetration into the cerebrospinal fluid.

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Oxytetracycline as a Factor in the Development of Growth and Weight of Children

OCTAVIO ANGELO DA VEIGA

Puriculture District, Rio de Janeiro, Brazil

Because of the excellent results on growth and development in underdeveloped children resulting from the administration of small doses of oxytetracycline, we decided to initiate a study in a group of children who could be closely controlled and supervised. A group of children was selected who had failed to develop and gain weight normally; these were "problem children" such as are always to be found in orphanages and similar institutions. Two groups were formed: group A consisting of 20 children who would receive oxytetracycline in dosages of 25 mg. twice daily, at luncheon and at dinner; group B of 20 children would serve as untreated controls.

The treatment and observation of the 40 children lasted for a period of three months. All subjects were weighed and measured once a month. Before initiating treatment, all the children were examined and those who were periodically subject to pharyngitis, tonsillitis, pyoderma were noted. During the course of the study, one of the Sisters in charge of the children observed that those children receiving oxytetracycline seemed to be healthier than the controls, showing less frequent illness than the latter group. During the course of study, all the children continued to live in the same environment, receiving the same attention and the same food. The members of the experimental group tolerated the drug perfectly, showing no disturbance of buccal mucosa, vomiting, or intestinal disturbances.

The 20 children in group A were given 25 mg. oxytetracycline in the crystalline, oral pediatric form twice daily for a period of three months. During this period, the treated group showed an average weight gain of 278 Gm. per month. These same children showed an average gain in height of more than 9 mm. (almost 1 cm.) per month. The 19 children in the control group B (of the original control group of 20, one left the asylum in September and the follow-up study could not be done) gained an average of 205 Gm. per month. The average increase in height was 7 mm. These data demonstrate that the gain in weight in group A who received oxytetracycline was 41 per cent higher than in the untreated control group B. Similarly, the children in group A showed a 40 per cent greater increase in height than the untreated controls.

In comparing the groups included in this study with the table of weight and height of Broman-Dahlberg and Lichtfustein, we note that of the 20 children, one showed weight gain similar to that of the table, two gained height in a similar fashion, while 19 gained less weight, and 18 gained less height. In the control group 7 children showed a greater gain, 4 a similar weight gain, and 9 lesser weight gain. In this connection, it should be recalled that the children of the oxytetracycline-treated group were those judged by the Mother Superior as being in the poorest condition prior to observation.

The favorable results of this study confirm the observation made by other investi-

gators on the administration of oxytetracycline to children in a dosage of 50 mg. per day for periods of months or even years. This regimen appears to be in no way harmful to the body (the indicated dosage for the treatment of infections is 25 to 40 mg./Kg./day). In a like manner, the current routine use of oxytetracycline in a balanced ration as animal feed (for poultry, pigs, horses,) has produced very good results on growth and development. These effects, particularly those observed in children who had previously shown lack of normal growth, suggest the possibility of adding oxytetracycline to the dietary formula of malnourished underweight children. The optimum dosage would require further study. Such addition could possibly be considered by manufacturers of children's dietary products. The development of such products could prove to be a solution for a large number of children who suffer from growth retardation, a condition frequently encountered in our pediatric clinics.

Subsequent to these observations made in August and September of 1958, we carried out a further study in 40 additional children during March-June, 1959. Twenty of these children received oxytetracycline with the other 20 serving as controls. The children took the oxytetracycline with pleasure, presumably because of the cherry flavor, and no untoward effects occurred in any of the 20 children who received a daily dosage of 50 mg. The nurses who cared for these children reported that they had less illness and reacted better than the controls not receiving oxytetracycline. At the beginning of treatment, the treated children were in notably poorer condition than the controls.

In this second study the 20 children receiving oxytetracycline showed a gain 37 per cent greater in weight and an increase in height 32 per cent greater than the untreated controls.

In light of these results we are applying a similar method to other patients treated in daily practice and have achieved good results during two months of therapy. Results will be published shortly.

In summary, I should like to point out that the children selected for oxytetracycline therapy were in poor condition (weight and height below that considered as the normal average in the table given by Fanconi). Of the 80 children included in the study, the 40 who received oxytetracycline showed a faster return toward normal weight and height than the untreated controls. These children, who received oxytetracycline eight months ago, today show a better state of health and have a relatively greater weight gain proportionately than the untreated controls. At present, a final conclusion should probably not be drawn. It is better merely to ask "What part has coincidence played in our results?" Our results are only indicative that oxytetracycline stimulated development in these children. This observation will require further research before a clear answer can be given or any true definitive conclusion reached.

The Effect of Antibiotics on a Spectrum of Tumors

KANEMATSU SUGIURA

*Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research,
New York, N. Y.*

Thirty years ago Fleming³⁷ isolated a substance with antimicrobial activity from a mold, *Penicillium notatum*. Since then many antibiotics have been isolated from culture filtrates of soil bacteria and Actinomycetales. According to Waksman, there are at present about 500 antibiotics known. Of these, only about 30 are in clinical use and still fewer, 1.0 per cent, demonstrate antitumor activity in human beings.

Experimental evidence obtained within the last few years has indicated that certain antibiotics have strong antitumor effects on certain animal neoplasms.^{1-5, 11-16, 18-21, 23, 24, 26-30, 32-36, 39, 41, 42, 44-48, 51-59, 61-65, 68-73, 75, 77-79, 81-88, 90, 91, 95-104, 106-115, 119-124, 131-136, 138-145, 147-150} Such studies continue to yield new information that may be applicable for possible trial against human cancer. ^{6-10, 17, 25, 31, 40, 43, 49, 50, 60, 66, 67, 74, 76, 80, 89, 92, 93, 94, 125-130, 137, 146}

It is well known that the responses of various tumors to a given agent may be strikingly different. Therefore, testing antibiotics against a spectrum of tumors may offer the possibility of detecting antitumor activities of antibiotics that may not be detected by screening when only one experimental tumor is used. Our observations on the effect of broad-spectrum antibiotics on a variety of transplantable mouse, rat, and hamster tumors are presented in the form of summary tables and discussed.

MATERIALS AND METHODS

The tumors used in the present study are listed in tables I and II. The history, biological properties, and cytological description of most of these tumors were presented previously.¹¹⁶⁻¹¹⁸ Except where otherwise noted, the melanoma was the pigmented form.

The methods employed in the chemotherapy studies were as follows: for the solid tumors,¹¹⁶ subcutaneous implantations of tumors (small pieces of tumor, each weighing approximately 6 mg.) into healthy young animals (18 to 22 Gm. mice, 80 to 100 Gm. rats and hamsters) were carried out by the usual trocar method with a single implant into the right axillary region. In every set of experiments, tumor-bearing animals were divided into two groups, one of controls and the other for treatment with antibiotics. The progress of the tumors in the animals was recorded graphically by measuring them in two diameters with calipers at weekly intervals for four weeks after tumor transplantation.

In the case of ascites tumors,¹⁰⁶ the intraperitoneal injection of 0.1 ml. of the fluid containing about 1 million cancer cells was made into each mouse in the

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TABLE I

Behavior of Transplantable Mouse Tumors in Animals

Tumor	Tumor designation	Required host strain	Per cent of tumor regression	Survival time of animals, weeks
Crocker sarcoma 180 (solid)	S-180	Swiss albino	5	2-3
Crocker sarcoma 180 (ascitic)	S-180 (ascitic)	Swiss albino	0	1-2
Lewis sarcoma T 241	T-241	C57 black	0	2-4
Sarcoma MA 387	MA-387	AKR	3	3-4
Ehrlich carcinoma (solid)	EC	Swiss albino	0	3-4
Ehrlich carcinoma (ascitic)	EC (ascitic)	Swiss albino	0	1-2
Bashford carcinoma 63	C-63	Swiss albino	5	3-6
Adenocarcinoma E 0771	E-0771	C57 black	2	3-4
Adenocarcinoma 755	755	C57 black	1	2-3
Adenocarcinoma RC	RC	DBA	0	2-3
Miyono adenocarcinoma	MC	AKR	4	3-5
Krebs 2 carcinoma (ascitic)	K-2 (ascitic)	Swiss albino	0	1-3
Carcinoma 1025	C-1025	AKR	2	4-7
Grand epidermoid carcinoma	Ep.C	Swiss albino	30	4-10
Lewis bladder carcinoma	BC	C57 black	0	2-4
Lewis lung carcinoma	LC	C57 black	3	2-4
Wagner osteogenic sarcoma	WOS	AKR	6	3-5
Ridgway osteogenic sarcoma	ROS	AKR	7	4-6
Patterson lymphosarcoma	PLS	AKR	2	2-4
Mecca lymphosarcoma	MLS	AKR	2	2-3
Gardner lymphosarcoma	GLS	C3H	3	2-3
Harding-Passey melanoma	H-PM	Swiss albino	5	9-12
Andervont Hepatoma	A-H	C	0	13-26
Glioma 26	G-26	C57 black	0	4-6
Friend virus leukemia	FVL	Swiss albino	10	8-12
Leukemia L 4946	L 4946	AKR	0	2-4

inguinal region from a $\frac{1}{4}$ ml. syringe fitted with a 25 gauge needle. These mice regularly develop large amounts of a milky ascites in 7 to 14 days and die in 10 to 16 days.

In the case of Friend virus leukemia,¹²¹ intraperitoneal injection of 0.2 ml. of a 10 per cent saline homogenate of leukemic spleens was made into each mouse in the inguinal region. This transplanted leukemia kills mice in two to four months with marked enlargement of liver and spleen. The effects of the antibiotics have

TABLE II

Behavior of Transplantable Rat and Hamster Tumors

Tumor	Tumor designation	Required host strain	Per cent of tumor regression	Survival time of animals, weeks
Flexner-Jobling carcinoma	FRC	Sherman	15	4-10
Walker carcinosarcoma 256	W-256	Sherman	3	2-5
Sarcoma R 39	R-39	Sherman	6	3-8
Jensen sarcoma	JRS	Sprague-Dawley	6	2-7
Murphy-Sturm lymphosarcoma	MRLS	Wistar	5	2-3
Iglesias functional ovarian tumor	FOT	AXC	0	8-22
Iglesias functional adrenal tumor	AT	AXC	0	16-26
Crabb hamster sarcoma	CHS	Golden syrian	0	2-4
Fortner hamster pancreas tumor	Pan. #1	Golden syrian	0	3-5
Fortner hamster intestine tumor	S.B. #1	Golden syrian	0	3-5

TABLE III

Effect of Antibiotics on Mouse Tumors

Tumor	Penicillin, 2000 mg./Kg./day	Streptomycin, 500 mg./Kg./day	Chlortetracycline, 50 mg./Kg./day	Chloramphenicol, 500 mg./Kg./day	Oxytetracycline, 150 mg./Kg./day
S-180	—	—	—	—	—
S-180 (ascitic)	—	—	—	±	±
T-241	—	—	—	—	—
MA-387	—	—	—	—	—
EC	—	—	—	—	—
EC (ascitic)	—	—	±	—	—
C63	—	—	—	—	—
E-0771	—	—	—	±	—
RC	—	—	—	—	—
MC	±	—	—	—	±
K-2 (ascitic)	—	—	—	—	±
C-1025	±	—	—	—	+
BC	—	—	—	—	—
LC	—	—	—	—	—
WOS	—	—	—	—	±
ROS	—	±	—	±	±
PLS	—	—	—	—	—
MLS	—	—	—	—	±
GLS	—	—	—	—	—
H-PM	—	—	—	—	±
G-26	±	—	—	—	+
FVL	—	—	—	—	—

been evaluated by comparison of the weights of spleens in treated and untreated infected mice three weeks after intraperitoneal injection of the leukemic spleen homogenate. At this time the spleens of leukemic mice weigh about 2.0 Gm., while those of normal female Swiss albino mice (about 8 weeks old) weigh about 0.18 Gm. For the convenience of routine screening purposes the three week observation period was chosen instead of the survival time. The animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water ad libitum.

Intraperitoneal injection of the antibiotics at maximum tolerated doses was begun 24 hours after inoculation with tumor material and was continued once daily for seven days. When, by this procedure, a substance caused a marked inhibition or destructive effect, it was tested on well-established 7 day old tumors.

TABLE IV

Effect of Antibiotics on Rat and Hamster Tumors

Tumor	Penicillin, 2000 mg./Kg./day	Streptomycin, 500 mg./Kg./day	Chlortetracycline, 50 mg./Kg./day	Chloramphenicol, 500 mg./Kg./day	Oxytetracycline, 150 mg./Kg./day
FRC	—	—	—	—	—
W-256	—	—	—	—	—
R-39	—	—	—	—	—
JRS	—	—	—	—	—
MRLS	—	—	—	—	—
CHS	—	—	—	—	±
Pan #1	—	—	—	—	—

The solvents used to dissolve the antibiotics were as follows: penicillin, streptomycin, oxytetracycline, azaserine, 6-diazo-5-oxo-L-norleucine, cycloheximide, puromycin, sarkomycin, kanamycin, mitomycin C, actinobolin, illudin M, streptovitamin A, alazopeptin, borrelidin, and sulfocidin in saline; concentrated solutions of actinomycin C, actinomycin D, actinomycin J, and pentamycin in ethanol were diluted in saline; carzinophilin in 1 per cent sodium borate and saline; chloramphenicol, fumagillin, gramicidin, netropsin, rossimycin A, rossimycin B, and griseofulvin in carboxymethylcellulose; and chlortetracycline in gum acacia or saline. Solutions of 6-diazo-5-oxo-L-norleucine, sarkomycin, carzinophilin, and fumagillin were prepared fresh daily. The usual injection volume of the various diluents was 0.5 ml. once daily. Sarkomycin, however, was given twice daily.

The degree of inhibition of growth of the solid tumors was graded according to the following scheme: — = No effect (tumor growth to three quarters or more of the diameter of the controls); \pm = slight inhibition (tumor growth from one half to three quarters of the diameter of the controls); + = moderate inhibition (tumor growth from one fourth to one half of the diameter of the controls); ++ = marked inhibition (failure to grow or growth to approximately one fourth of the average diameter of the controls); +++ = complete inhibition or destruction of tumors.

The degree of inhibition of ascites tumor growth was graded according to the following scheme: — = No effect (marked abdominal distention; the fluid volume increased to three fourths or more of the controls, 10 to 25 ml. ascites); \pm = slight inhibition (moderate abdominal distention; the fluid volume increased to about one half the volume of the controls, 5 to 9 ml. ascites); + = moderate inhibition (slight abdominal distention; the fluid volume increased to about one fourth the volume of the controls, 1 to 4 ml. ascites); ++ = marked inhibition (no abdominal distention; no gross ascites); +++ = complete inhibition or destruction of ascites.

The degree of inhibition of virus leukemia was graded according to the following scheme: — = No effect (spleen weight from three quarters or more of the average spleen weight of the leukemic spleens, controls); \pm = slight inhibition (spleen weight from one half to three quarters of the average spleen weight of the controls); + = moderate inhibition (spleen weight from one fourth to one half of the average spleen weight of the controls); ++ = marked inhibition (spleen weight approximately one fifth of the average spleen weight of the controls); +++ = complete inhibition (no alteration of spleen weight from normal, nonleukemic animals).

RESULTS

The results obtained with antibiotics tested at or near maximum tolerated doses are summarized below and in tables III to XII. Test groups consisted of 5 or 10 animals, and each experiment was repeated; the data presented are averages of the results of multiple experiments. Evaluation of effects has been based on results observed two weeks after tumor inoculation, except for the Friend virus leukemia. Because of the long survival time of these leukemic mice, observations were made after three weeks.

From the tabulated data in tables III and IV it is evident that at well-tolerated doses none of the more widely used antimicrobial antibiotics (penicillin, strepto-

TABLE V
Effect of Antibiotics on Mouse Tumors

Tumor	Mitomycin C, 2.0 mg./Kg./day	Actinobolin, 1000 mg./Kg./day	Actinomycin D, 0.025 mg./Kg./day	Kanamycin, 500 mg./Kg./day	Pentamycin, 1.0 mg./Kg./day
S-180	±	—	—	—	—
S-180 (ascitic)	+++	+++	+++	—	—
T-241	±	—	—	—	—
MA-387	±	—	—	—	—
EC	±	±	—	—	—
EC (ascitic)	+++	+++	+++	—	—
C-63	+	—	—	—	—
E-0771	++	+	+	±	±
MC	++	±	—	—	—
K-2 (ascitic)	—	—	+++	—	—
C-1025	+++	++	++	—	—
BC	±	—	—	—	—
LC	+	—	—	—	—
WOS	+	+	—	—	—
ROS	+++	—	+++	—	—
MLS	—	—	—	—	—
GLS	—	—	—	—	—
H-PM	++	—	±	—	—
G-26	++	++	—	—	—
FVL	+++	±	—	—	±
L-4946	—	—	—	—	—

— = No effect; ± = slight inhibition; + = moderate inhibition; ++ = marked inhibition; +++ = complete inhibition or destruction of tumors.

mycin, chloramphenicol, chlortetracycline, and oxytetracycline) has shown a significant activity in the large groups of mouse, rat, and hamster tumors we have tested. These negative results with the more readily available antibiotics furnish background information of value in the future evaluation of antibiotic-containing filtrates from cultures of microorganisms. These data may also provide some comfort, though not complete assurance, that there is little harmful influence on the growth of tissue exposed to some of these antibiotics. It has been assumed, or perhaps hoped, that such antibiotics when added to tissue culture media, to preparations of compounds used in chemotherapy studies, and to physiological saline used to moisten tumor fragments prior to transplantation have not adversely affected the tumor material treated in this manner.

TABLE VI
Effect of Antibiotics on Rat and Hamster Tumors

Tumor	Mitomycin C, 1.0 mg./Kg./day	Actinobolin, 1000 mg./Kg./day	Actinomycin D, 0.025 mg./Kg./day	Kanamycin, 500 mg./Kg./day	Pentamycin, 1.0 mg./Kg./day
FRC	+++	±	—	—	—
W-256	+++	++	—	—	—
JRS	+++	±	±	—	—
MRLS	++	±	—	—	—
FOT	+++	—	—	—	—
AT	+	—	—	—	—
CHS	++	±	—	—	—
Pan. #1	—	—	—	—	—
S.B. #1	—	±	±	—	—

It is clear that, among the antibiotics with antitumor activity, there are differences in effectiveness against the various tumors (tables V and VI). This emphasizes the observations made previously with various synthetic compounds and thus suggests again the potential utility of the tumor spectrum as a means for evaluating new chemotherapeutic agents.

Because mitomycin C had a definite inhibitory effect on 16 of 25 solid tumors, a comparison of its antitumor activity with that of four other antibiotics might be of interest. These are pentamycin, actinobolin, actinomycin D, and kanamycin, all isolated from strains of *Streptomyces*. From the tabulated data in tables V and VI it is evident that antitumor activity of actinobolin and actinomycin D was essentially the same with the exception that actinobolin had a marked inhibitory effect on glioma 26 and Walker carcinosarcoma 256; actinomycin D had no inhibitory effect on these tumors. On the other hand, actinomycin D had a destructive action on Ridgway osteogenic sarcoma, whereas actinobolin was inactive.

New antifungal antibiotics, kanamycin and pentamycin, demonstrated no inhibitory effect on any of the 24 tumors, including two ascites tumors and one virus leukemia. Mitomycin C, isolated from *Streptomyces caespitosus*⁴⁵ showed a marked to complete inhibitory effect on 15 of 29 various tumors, including solid and ascites tumors.

In tumor chemotherapy screening studies, the choice of the test tumors is a major consideration, for it is not practical at present to conduct a large screening program in which three or more tumors are used routinely. It has been realized that chemicals and antibiotics effective against one tumor would not necessarily be effective against other tumors. Accordingly, we have supplemented our chemotherapy screening program¹¹⁶ with an examination of a variety of tumors—a tumor spectrum study—in order to test compounds of unusual theoretical interest in addition to those with some demonstrated antitumor activity. The present report will compare, in the spectrum of mouse, rat, and hamster tumors, the effects of O-diazoacetyl-L-serine (azaserine) and its analogue 6-diazo-5-oxo-L-norleucine, which has been one of the few compounds showing a marked inhibition of sarcoma 180^{23, 24, 102, 119, 132} in mice. These antibiotics were isolated from the culture media of *Streptomyces* and are now synthesized. From tables VII and VIII, it can be observed that the analogue is capable of inhibiting some of the tumors. A comparison of the effectiveness of azaserine and the analogue against our tumor spectrum indicated the superiority of the analogue over azaserine on mouse tumors and an inferiority against rat tumors. As in the case of azaserine, the analogue was less effective on 7 day old tumors than 1 day old tumors.

Cycloheximide, isolated from the culture media of a *Streptomyces* strain, was tested on a spectrum of tumors at a dosage of 25 mg./Kg./day for mice and 1.0 mg./Kg./day for rats and hamsters. It is apparent from the data in tables VII and VIII that cycloheximide had a marked inhibitory effect on Miyono adenocarcinoma and Mecca lymphosarcoma and a moderate inhibitory effect on Bashford carcinoma 63, adenocarcinoma E 0771, carcinoma 1025, Wagner and Ridgway osteogenic sarcomas, Harding-Passey melanoma, sarcoma 180 ascites tumor, Ehrlich ascites carcinoma, and Flexner-Jobling carcinoma, but 15 other tumors were either only slightly inhibited or unaffected.

Tables VII and VIII also show that repeated intraperitoneal injections of puromy-

TABLE VII

Effect of Antibiotics on Mouse Tumors

Tumor	Azaserine, 5.0 mg./Kg./day	6-Diazo-5-oxo- L-norleucine, 0.1 mg./Kg./day	Cycloheximide, 25 mg./Kg./day	Puromycin, 100 mg./Kg./day	Fumagillin, 15 mg./Kg./day
S-180	++	+	—	—	±
S-180 (ascitic)	+	++	+	—	+
T-241	±	—	±	—	+
MA-387	—	±	±	±	++
EC	±	+	—	±	+
EC (ascitic)	+	++	+	—	+++
C-63	—	+	+	+	+
E-0771	+	±	+	±	++
MC	±	++	++	+	++
K-2 (ascitic)	+	++	—	—	—
C-1025	—	++	+	+++	++
BC	—	±	±	+	+
LC	—	—	±	—	+
WOS	±	—	+	—	++
ROS	—	++	+	+	++
PLS	+	—	—	—	—
MLS	+	++	++	—	++
GLS	±	—	±	—	++
H-PM	±	—	+	+	+
G-26	—	±	±	—	++
FVL	+	—	—	—	—
L-4946	—	—	—	—	—

cin, isolated from *Streptomyces*, at the maximum tolerated dosage of 100 mg./Kg./day for mice and 50 mg./Kg./day for rats and hamsters had a destructive effect on carcinoma 1025 and a moderate inhibitory effect on Bashford carcinoma 63, Miyono adenocarcinoma, Ridgway osteogenic sarcoma, and Harding-Passey melanoma, but it had practically no inhibitory effect on other tumors, including ascites tumors.

The present study indicates that fumagillin, an antibiotic produced by *Aspergillus fumigatus*, was one of the most effective of the 29 antibiotics tested. Daily maximum tolerated dosages of 15 mg./Kg. of fumagillin had a destructive effect on Ehrlich ascites carcinoma and a marked inhibitory effect on sarcoma MA 387, adenocarcinoma E 0771, Miyono adenocarcinoma, carcinoma 1025, Wagner and

TABLE VIII

Effect of Antibiotics on Rat and Hamster Tumors

Tumor	Azaserine, 2.5 mg./Kg./day	6-Diazo-5-oxo- L-norleucine, 0.05 mg./Kg./day	Cycloheximide, 1.0 mg./Kg./day	Puromycin, 50 mg./Kg./day	Fumagillin, 5.0 mg./Kg./day
FRC	±	—	+	±	++
W-256	++	±	±	±	+
JRS	+	—	±	±	++
MRLS	+	—	±	±	++
CHS	+	—	—	±	++
Pan. #1	—	—	—	±	—
S.B. #1	—	—	—	—	—

Ridgway osteogenic sarcomas, Mecca and Gardner lymphosarcomas, and glioma 26. It had a moderate inhibitory effect on seven other mouse tumors. It is interesting to note that daily dosages of 5.0 mg./Kg. of fumagillin in rats and hamsters had a marked inhibitory effect on Flexner-Jobling carcinoma, Jensen sarcoma, Murphy-Sturm lymphosarcoma, and Crabb hamster sarcoma. However, there was no great inhibition of any of the mouse, rat, and hamster tumors when tests at maximum tolerated dosages of fumagillin were made with 7 day old growths.

Streptovitamin A at daily dosages of 0.3 mg./Kg. had a marked inhibitory effect on Wagner osteogenic sarcoma, Flexner-Jobling carcinoma, Walker carcinosarcoma 256, and Crabb hamster sarcoma (tables IX and X).

Sarkomycin (250 mg./Kg./day), carzinophilin (2500 units/Kg./day), and alazopeptin (0.05 mg./Kg./day), all isolated from *Streptomyces*, had a destructive effect on all three ascites tumors. Similar results were obtained even when the agents were administered subcutaneously. There was almost no inhibitory effect on all solid tumors tested, except for a marked inhibitory effect on carcinoma 1025 and Crabb hamster sarcoma by sarkomycin, a moderate inhibitory effect on Harding-Passey melanoma by carzinophilin, and a moderate inhibitory effect on sarcoma 180, carcinoma 1025, and Wagner osteogenic sarcoma by alazopeptin (tables IX and X).

Netropsin (tables XI and XII), at a dosage of 25 mg./Kg./day for seven days, had a destructive effect on Friend virus leukemia, a marked inhibitory effect on Wagner osteogenic sarcoma, a moderate inhibitory effect on sarcoma MA 387,

TABLE IX
Effect of Antibiotics on Mouse Tumors

Tumor	Sarkomycin, 250 mg./Kg./day	Carzino- philin, 2500 units/Kg. /day	Actinomycin C, 0.025 mg./Kg./day	Actinomycin J, 0.025 mg./Kg./day	Strepto- vitacin A, 0.3 mg./Kg./day	Alazopeptin, 0.05 mg./Kg./day
S-180	—	—	—	—	+	+
S-180 (ascitic)	+++	+++	+++	+++	—	+++
T-241	±	—	—	±	—	±
MA 387	—	—	—	—	—	±
EC	±	—	—	±	—	+
EC (ascitic)	+++	+++	+++	+++	±	+++
C-63	—	—	—	—	—	—
E-0771	—	—	—	±	+	±
MC	—	±	±	+	—	±
K-2 (ascitic)	+++	+++	—	+++	—	—
C-1025	++	±	++	++	+	+
BC	—	±	—	—	—	—
LC	—	±	—	±	±	—
WOS	±	±	++	±	++	+
ROS	—	±	+++	++	±	±
MLS	—	—	—	—	—	±
GLS	—	—	—	—	—	—
H-PM	±	+	±	—	—	—
G-26	—	—	±	+	—	—
FVL	—	—	+	—	±	±
L-4946	—	—	—	—	—	±

TABLE X

Effect of Antibiotics on Rat and Hamster Tumors

Tumor	Sarkomycin, 250 mg./Kg./day	Carzino- philin, 2500 units/Kg. /day	Actinomycin C, 0.025 mg./Kg./day	Actinomycin J, 0.025 mg./Kg./day	Strepto- vitacin A, 0.3 mg./Kg./day	Alazopeptin, 0.05 mg./Kg./day
FRC	—	—	—	±	++	—
W-256	—	—	±	±	++	—
JRS	—	±	—	+	+	—
MRLS	—	—	±	±	—	—
CHS	++	±	++	++	++	—
Pan. #1	—	—	—	—	—	—
S.B. #1	±	—	—	±	—	—

Ehrlich carcinoma, Harding-Passey melanoma, and leukemia L 4946, but it had practically no inhibitory effect on 19 other tumors, including ascites tumors.

Gramicidin (12.5 mg./Kg./day) had a marked inhibitory effect on adenocarcinoma E 0771 and a moderate inhibitory effect on Murphy-Sturm lymphosarcoma, but this antibiotic had almost no inhibitory effect on other tumors (tables XI and XII).

Sulfocidin (0.2 mg./Kg./day) had a destructive effect on sarcoma 180 ascites tumor and Ehrlich ascites carcinoma. There was almost no inhibitory effect on solid tumors (tables XI and XII).

Repeated injections of 2.0 mg./Kg./day of borrelidin had practically no inhibitory effect on 28 tumors tested in the mouse, rat, and hamster (tables XI and XII).

TABLE XI

Effect of Antibiotics on Mouse Tumors

Tumor	Netropsin, 25 mg./Kg./day	Gramicidin, 12.5 mg./Kg./day	Sulfocidin, 0.2 mg./Kg./day	Borrelidin, 2.0 mg./Kg./day	Illudin M, 0.5 mg./Kg./day	Griseofulvin, 500 mg./Kg./day
S-180	—	—	—	±	—	—
S-180 (ascitic)	—	—	+++	±	—	—
T-241	—	—	—	—	—	—
MA-387	+	—	—	—	—	—
EC	+	—	—	—	±	—
EC (ascitic)	—	—	+++	—	—	—
C-63	—	±	—	—	±	—
E-0771	—	++	±	—	±	±
MC	—	±	—	—	—	—
C-1025	±	—	—	±	++	—
BC	—	±	±	—	—	—
LC	±	±	—	—	—	—
WOS	++	±	—	—	±	—
ROS	±	±	±	—	—	++
MLS	—	—	—	—	—	—
GLS	—	—	—	—	±	—
H-PM	+	—	±	—	+	+
G-26	—	—	—	±	±	—
FVL	+++	—	—	—	—	—
L-4946	+	—	—	—	—	—

TABLE XII

Effect of Antibiotics on Rat and Hamster Tumors

Tumor	Netropsin, 15 mg./Kg./day	Gramicidin, 12.5 mg./Kg./day	Sulfocidin, 0.2 mg./Kg./day	Borrelidin, 0.5 mg./Kg./day	Illudin M, 0.5 mg./Kg./day	Griseofulvin, 500 mg./Kg./day
FRC	—	—	±	±	—	
W-256	±	±	—	—	—	
JRS	±	—	—	—	—	—
MRLS	—	+	—	—	—	—
FOT	—					
AT	—					
CHS	—	—	—	±	—	
Pan. #1	—	—		—	—	
S.B. #1	—	—		—	—	

Illudin M at 0.5 mg./Kg./day had a marked inhibitory effect on carcinoma 1025 and a moderate inhibitory effect on Harding-Passey melanoma, but it had practically no inhibitory effect on 19 other tumors (tables XI and XII).

Rossimycin A, at a dosage level of 0.025 mg./Kg./day for seven days, had a marked inhibitory effect on Ridgway osteogenic sarcoma, but rossimycin B, at a dosage of 1.0 mg./Kg./day, had no inhibitory effect on Ridgway osteogenic sarcoma.

Experiments with actinomycin D (an American product) were repeated with crystalline actinomycin J (a Japanese product) and actinomycin C (a German product). The results obtained with our spectrum of tumors are summarized in tables IX and X. The carcinolytic actions of actinomycin J, actinomycin C, and actinomycin D were essentially the same. Daily maximum tolerated dosages of 0.025 mg./Kg. of actinomycin J had a complete destructive effect on all three ascites tumors but had practically no inhibitory effect on solid tumors, except for a marked inhibitory effect on carcinoma 1025, Ridgway osteogenic sarcoma, and Crabb hamster sarcoma and a moderate inhibitory effect on Miyono adenocarcinoma, glioma 26, and Jensen sarcoma.

Repeated intraperitoneal injections of 0.025 mg./Kg./day of actinomycin D had a marked inhibitory effect on nonpigmented Harding-Passey melanoma, but only a slight inhibitory effect on pigmented Harding-Passey melanoma. Fumagillin had a marked inhibitory effect on nonpigmented melanoma and a moderate inhibitory effect on pigmented melanoma. Carzinophilin had a slight inhibitory effect on non-

TABLE XIII

Approximate Per Cent of 7 Day Old Mouse and Rat Tumors Cured by Antibiotics

Antibiotic administered	Dosage, mg./Kg./day	Tumor	Number treated	Number cured	Per cent cured
Actinomycin D	0.025	ROS	120	96	80
Mitomycin C	2.0	C1025	45	38	84
Mitomycin C	2.0	ROS	50	43	86
Mitomycin C	2.0	FVL	40	20	50
Mitomycin C	1.0	FRC	50	50	100
Mitomycin C	1.0	JRS	50	50	100

pigmented melanoma but it had a moderate inhibitory effect on pigmented melanoma. 6-Diazo-5-oxo-L-norleucine had no inhibitory effect on Harding-Passey melanoma, both pigmented and nonpigmented forms.

Both azaserine and borrelidin had only a slight inhibitory effect on skin cancers, carcinoma 1025, and Grand epidermoid carcinoma.

Azaserine had a moderate inhibitory effect on Andervont hepatoma.

In recent years, ascites tumors have been used to a considerable extent by investigators in chemotherapy studies. These tumors provide an easy challenge to chemotherapeutic agents because the intraperitoneal injection of compounds against the intraperitoneal ascites form of tumor is, in effect, an *in vivo*-*in vitro* test. Thus it is possible to obtain an idea of the action of a compound without as great a degree of intervention by various organs. A comparison of carcinostatic effects of 27 antibiotics on both ascites and solid forms of the same mouse tumors has demonstrated that the ascites forms appear to be a more sensitive system for the detection of antitumor activity. Mitomycin C, fumagillin, actinomycin C, actinomycin D, actinomycin J, actinobolin, sarkomycin, carzinophilin, sulfocidin, and alazopeptin had a destructive effect on both sarcoma 180 and Ehrlich carcinoma ascites tumors, but these antibiotics had practically no inhibitory effect on the respective solid tumors except fumagillin and alazopeptin, which had a moderate inhibitory effect on the respective solid tumors. Intraperitoneal injection of chemicals and antibiotics into ascites tumor-bearing mice, however, represents a form of intratumoral treatment and, as such, has less significance than treatment of solid tumors growing at a distance from the injection site.

Among the antibiotics with antitumor activity, there are differences in effectiveness against the various tumors. This emphasizes the observations made previously with various synthetic compounds and thus suggests again the potential utility of the tumor spectrum. For detailed studies there may be selected from a group of tumors the one that shows the most useful response. It is evident from the response of various tumors to actinomycin D and mitomycin C that the Ridgway osteogenic sarcoma and carcinoma 1025 would be useful tools. These tumors are retarded markedly in their growth by doses of actinomycin D and mitomycin C that are not toxic to the host. Actinomycins C, D, and J, mitomycin C, fumagillin, azaserine analogue, griseofulvin, and rossimycin A had from a marked to complete inhibitory effect on Ridgway osteogenic sarcoma. Actinomycins C, D, and J, mitomycin C, fumagillin, azaserine analogue, actinobolin, puromycin, sarkomycin, and illudin M had a similar antitumor action on carcinoma 1025.

It is extremely interesting that repeated injections of mitomycin C, fumagillin, and actinobolin had a marked inhibitory effect on glioma 26 (a brain tumor). We have tested more than 250 synthetic compounds with this tumor and found that only the following five compounds caused a marked inhibitory effect: purine-6-thiogluco-side (250 mg./Kg./day), 6-mercaptopurine riboside (125 mg./Kg./day), 3,3-dimethyl-1-*p*-nitrophenyl triazene (125 mg./Kg./day), 3-cyclohexyl-3-methyl-1-*p*-tolyl triazene (500 mg./Kg./day), and 3-(*p*-acetoxyphe-nyl)-4-(2-thienyl)-3-hexene (7.5 mg./Kg./day). However, no antibiotic or compound caused complete destruction of glioma 26.

Azaserine was the only antibiotic among 29 purified or crystalline antibiotics we tested that had a marked inhibitory effect on sarcoma 180. However, azaserine

analogue, streptovitacin A, and alazopeptin had a moderate inhibitory effect on sarcoma 180.

Sarcoma MA 387 was most sensitive to fumagillin and then netropsin, while this tumor was practically insensitive to 22 other antibiotics tested.

It is interesting to note that actinomycin D, actinomycin J, mitomycin C, azaserine analogue, and puromycin had a destructive effect or marked inhibitory effect on Ridgway osteogenic sarcoma but no inhibitory effect on the Wagner osteogenic sarcoma except for mitomycin C. Both tumors arose spontaneously in AKR mice.¹¹⁶ Transplants of these tumors grow equally rapidly in AKR mice and kill animals in three to four weeks. Morphologically these tumors are similar. However, the alkaline phosphatase content of Ridgway osteogenic sarcoma is less than that of Wagner osteogenic sarcoma, about 20 units of alkaline glycerophosphatase per Gm. against about 50 units. Sarcoma 180 and other solid tumors contained from 0.1 to 0.2 units of alkaline glycerophosphatase per Gm. of fresh tissues.

Mitomycin C, fumagillin, and gramicidin had a marked inhibitory effect on mammary adenocarcinoma E 0771. Mitomycin C, fumagillin, azaserine analogue, and cycloheximide had a marked inhibitory effect on another mammary tumor, Miyono adenocarcinoma.

Azaserine analogue, cycloheximide, and fumagillin had a marked inhibitory effect on Mecca lymphosarcoma, but only mitomycin C among 29 antibiotics tested had a marked inhibitory effect on Harding-Passey melanoma (pigmented form). We have tested more than 600 other compounds with this melanoma, and they caused only slight or no inhibitory effect, except for triethylene thiophosphoramide.¹¹⁸

Previously, it has been found in our laboratories that sarcoma 180 has been "cured" by 6-mercaptopurine,^{22,105} that carcinoma 1025 has been "cured" by 3-bis (β -chloroethyl) aminomethyl-4-methoxymethyl-5-hydroxy-6-methyl pyridine,¹¹⁶ by triethylene melamine,¹¹⁷ and by mitomycin C,¹¹⁰ and now Ridgway osteogenic sarcoma has been "cured" by actinomycin D and mitomycin C. These results encourage the hope that more chemicals and antibiotics may be found that have a destructive action specific for different tumors.¹⁰⁸

Although as yet there is no evidence that human cancers are caused by viruses, much experimental evidence indicates that viruses may be the cause of neoplastic growth. About three years ago a new transplantable mouse virus leukemia³⁸ was added to our spectrum of tumors. Among 200 selected compounds and antibiotics tested¹¹² against the Friend mouse virus leukemia, mitomycin C, triethylene melamine, thioguanine, myleran, 1,9-di (methanesulfonyl) nonane, 9 α -fluoro-2 α -methyl-hydrocortisone acetate, estradiol, and netropsin showed a complete inhibitory effect on this virus leukemia.

In connection with the *in vivo* investigations, a study was made to test the possible direct antiviral action of mitomycin C *in vitro*. Results showed that 100 and 500 γ /ml. of mitomycin C for 24 hours' incubation at 4 to 5 C. had no inhibitory effect, whereas 1000 γ /ml. had only a slight inhibitory effect on the subsequent development of leukemia. It is interesting to note that the transplantability of both Ehrlich ascites carcinoma and sarcoma 180 ascites tumor, which are not virus tumors, was completely destroyed by exposure to 10 γ /ml. and 1 γ /ml. of mitomycin C, respectively, for 24 hours at 4 to 5 C.

Since the Friend mouse virus leukemia responded well to treatment with mito-

mycin C, we tested its antitumor activity in another virus tumor, Rous chicken sarcoma. Results showed that subcutaneous or intraperitoneal injections of 2.0 mg./Kg./day of mitomycin C for seven days had a marked inhibitory effect on the growth of Rous chicken sarcoma in young chickens, but 1.0 mg./Kg./day of mitomycin C had practically no effect. Daily maximum tolerated dosages of 0.5 mg./Kg. of triethylene melamine, 20 mg./Kg. of busulfan, and 50 mg./Kg. of 1, 9-di (methane-sulfonyl) nonane had only a slight inhibitory effect on Rous chicken sarcoma. Against Friend virus leukemia, however, these compounds showed a maximum inhibitory effect equal to that of mitomycin C. In vitro experiments with Rous chicken sarcoma showed that 100, 500, and 1000 γ /ml. of mitomycin C for 24 hours' incubation at 4 to 5 C. had practically no inhibitory effect on the subsequent growth of the virus tumor.

Daily subcutaneous or intraperitoneal injections of fumagillin (5.0 mg./Kg.), nitrogen mustard (0.25 mg./Kg.), sarkolysin (1.0 mg./Kg.), triethylene melamine (0.25 mg./Kg.), and cytoxan (50 mg./Kg.) for seven days had no inhibitory effect on the growth of Rous sarcoma no. 1 in chickens, but cortisone (37.5 mg./Kg.) had a moderate inhibitory effect.

EFFECT OF ANTIBIOTICS ON WELL-ESTABLISHED TUMORS

The success of several antibiotics in producing damage in 1 day old tumors of the mouse and of the rat led to the more rigorous test on well-established 7 day old tumors. The results obtained from these experiments are summarized in table XIII. Our study showed that it was possible to cause the permanent and complete regression of certain well-established 7 day old tumors of Ridgway osteogenic sarcoma by actinomycin D and of carcinoma 1025, Ridgway osteogenic sarcoma, Friend virus leukemia, Flexner-Jobling carcinoma, and Jensen sarcoma by mitomycin C.

RESULTS WITH SPONTANEOUS MAMMARY TUMORS

Because mitomycin C had a distinct inhibitory effect on the growth of three transplantable mammary carcinomas, it was tested in a total of 96 Swiss albino mice with recently developed spontaneous mammary adenocarcinomas. In various groups of these mice, intraperitoneal injections of mitomycin C (1 to 1.5 mg./Kg./day for 10 to 14 days) had a moderate inhibitory effect on spontaneous breast tumors, and while intravenous injections of the antibiotic had a marked inhibitory effect, there was no significant increase in tumor regression over the 5 per cent observed in the controls.

In the course of the investigation, actinomycin D and fumagillin were used to treat 50 C3H mice with small spontaneous breast tumors. The tumor-bearing animals were injected intraperitoneally with 0.025 mg./Kg. of actinomycin D or 15 mg./Kg. of fumagillin once daily for 7 to 14 days. The results showed these antibiotics had practically no inhibitory effect on spontaneous mammary cancers in C3H mice.

SUMMARY

This progress report notes the cure of some transplantable animal tumors by antibiotics, namely, mitomycin C and actinomycin D. The effectiveness, at least

temporarily, of these antibiotics against human neoplasms makes one hopeful of the eventual attainment of our goal, the cure of cancer in man.

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Diazomycins A, B, and C, Three Antitumor Substances

I. Isolation and Characterization

KOPPAKA V. RAO, SAMUEL C. BROOKS, JR., MAX KUGELMAN,
AND A. A. ROMANO

Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

Antitumor substances with an aliphatic diazo functional grouping have assumed a unique and important role in recent years. Not only have they shown, as a group, high antitumor activity in experimental animals, but they also represent a novel type among natural products. Three such compounds have been described in the past: azaserine,¹ 6-diazo 5-oxo norleucine,² and alazopeptin³ but the list promises to be a long one.

In our Microbiology Department an actinomycete culture was isolated, the broth filtrates of which showed a high degree of inhibition* against sarcoma 180 and adenocarcinoma 755 and moderate activity against leukemia 1210 in mice. This culture was characterized as *Streptomyces ambofaciens* and its description will appear elsewhere.

Preliminary experiments toward the isolation of the active principle showed that the latter is not readily extractable into common organic solvents and is not adsorbed on or eluted from several types of carbons or ion exchange resins. It showed extreme sensitivity to acids and appeared to be most stable in the pH range 6.0 to 8.0. The broths also showed some degree of activity toward yeasts and selected bacteria grown in suitable artificial media. These microbiological methods, especially activity against *Bacillus subtilis*, were used throughout for following the progress of isolation.

As a first step in the recovery procedure, the filtered broth is concentrated to 5 to 10 per cent of the original volume, added to 10 volumes of methanol, filtered, and the filtrate reconcentrated. This is then passed through a column of Darco and the crude active material eluted with 5 to 10 per cent aqueous acetone.

The product at this stage showed definite indications of being an aliphatic diazo compound, and in particular a diazoketone. Paper chromatography (system: 80 per cent aqueous isopropanol) showed two or three active components. A generic name of diazomycin was proposed to designate these components and attempts were directed toward their separation. In order to follow the progress of the separation, both microbiological and spectrophotometric methods were used. For the latter, an aliquot is diluted with water and with 1 *N* acid and the difference in their optical densities at 275 mμ was read. This difference was taken to be proportional to the concentration of the diazo compound. Ratio of the optical density to microbiological activity appeared to differ from one component to the other and was used for distinguishing purposes.

The crude starting material is first passed through a column of a strong base type anion exchange resin such as Dowex-1 in its acetate form. About 25 to 45

* Testing carried out under Cancer Chemotherapy National Service Center Screening Program, Contract No. SA-43-ph-1926.

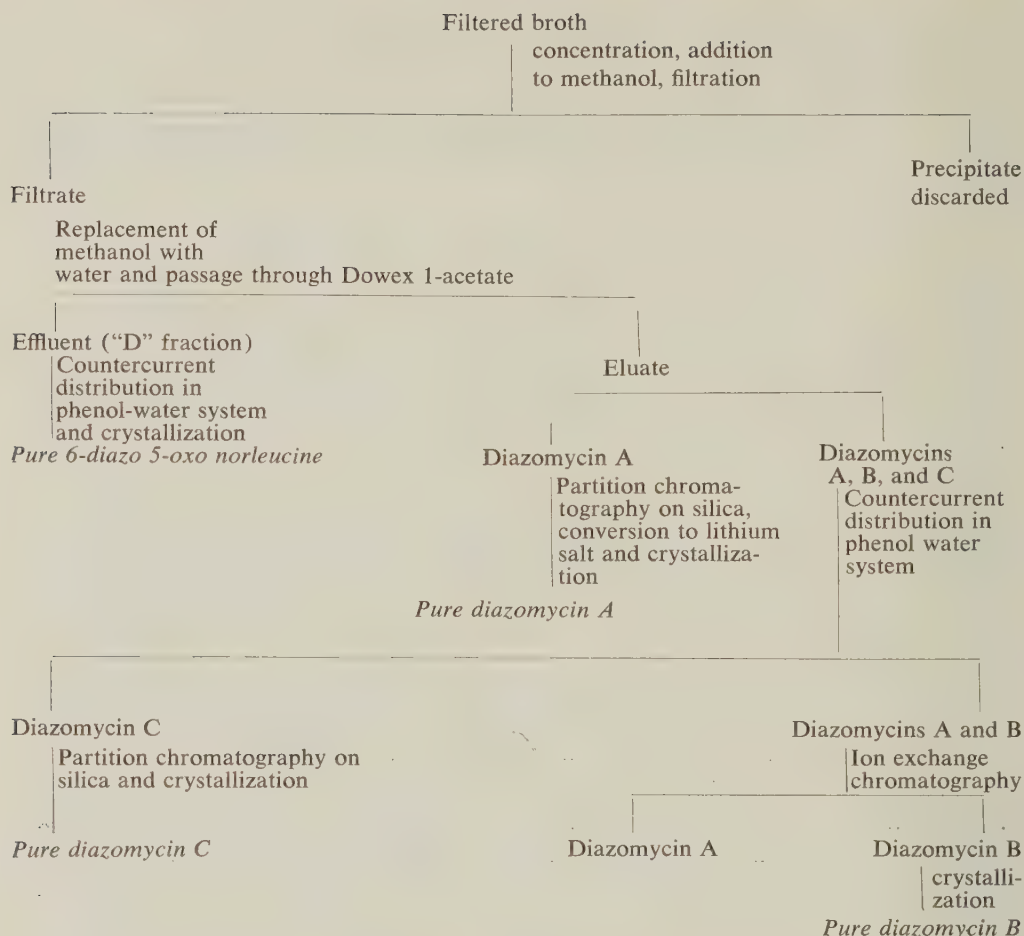


FIG. 1. Scheme for the separation and purification of diazomycins.

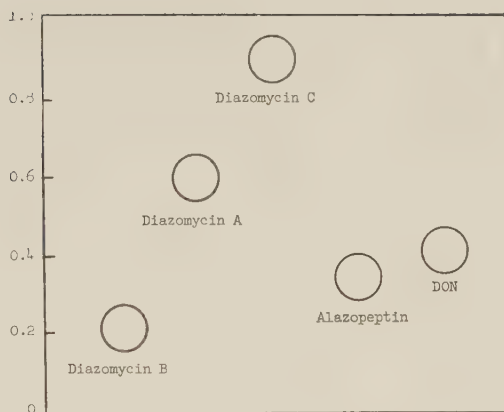
per cent of the total diazo compound appeared in the effluent. This was designated as the "D" fraction and will be dealt with later. When the column was eluted with 1 per cent phosphate buffer (pH 7.0) a partial separation into two fractions took place. The major fraction, diazomycin A comprised about 40 to 50 per cent of the total and the second one diazomycin B accounted for the rest.

Purification of diazomycin A was next carried out by partition chromatography on Celite or silica gel with the system: phosphate buffer, *n*-butanol, and isopropanol. The product was recovered from the appropriate fractions, converted into its lithium salt and crystallized as such.

The "B" fraction which still contained appreciable amounts of "A" as well as several other impurities was first subjected to countercurrent distribution between phenol and water. The distribution showed a single peak for both the "A" and "B" components when followed by microbiological procedure. However, when tested by the spectrophotometric method a new peak appeared, well separated from the "A-B" peak. This new fraction was designated diazomycin C.

Separation of "B" and "A" was considerably more difficult by either distribution or partition chromatography procedures. The latter method invariably decomposed

FIG. 2. Paper chromatography of the diazomycins.



the "B" fraction even under very mild conditions. Complete separation could, however, be achieved by ion exchange chromatography on Dowex-1 resin in its acetate form. When eluted slowly with dilute phosphate buffer the two are separated not only from each other but also from several other persistent impurities. Diazomycin B was recovered from the appropriate fractions, freed from the inorganic impurities by methanol extraction, and crystallized from aqueous methanol.

The "C" component was recovered from the distribution and purified by the partition chromatography procedure similar to the one used in connection with diazomycin A. The purified material was crystallized from ethanol.

The "D" fraction after two passages through countercurrent distribution between phenol and water was of sufficient purity to be crystallized from methanol. Properties of the crystalline solid showed that it is identical with 6-diazo 5-oxo norleucine.

The isolation procedure already described could be somewhat simplified by substituting the initial carbon adsorption step by the ion exchange procedure with Dowex-1 acetate. This has advantages in that it eliminates in the effluent the known compound, 6-diazo 5-oxo norleucine at an early stage and separates the bulk of the diazomycin A from the minor products diazomycins B and C. The scheme shown in figure 1 summarize the procedures used for the separation and purification of the several diazomycins.

The behavior of all the four components in paper chromatography, counter-

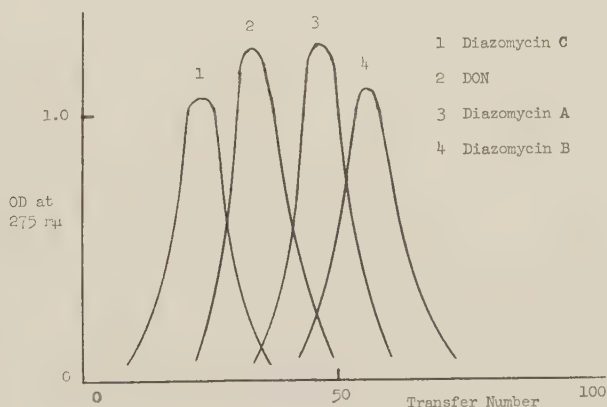


FIG. 3. The countercurrent distribution of diazomycins.

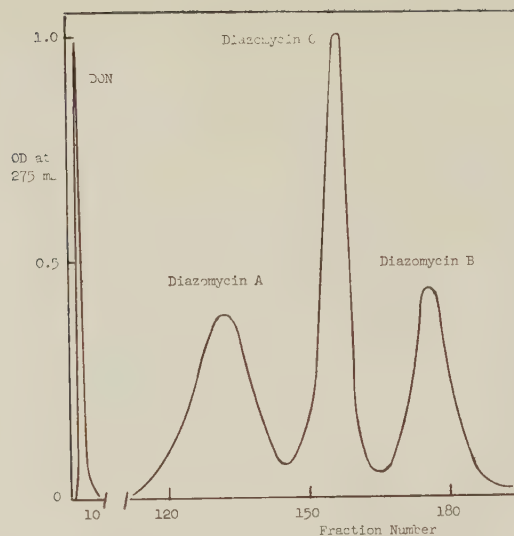


FIG. 4. Ion exchange chromatography of diazomycins.

current distribution in phenol-water system, and in the ion exchange chromatography is shown in figures 2, 3, and 4. The ultraviolet and infrared spectra of diazomycins A, B, and C are shown in figures 5 to 8. A comparison of some of the properties of diazomycins A, B, and C is shown in table I.

EXPERIMENTAL

The culture is grown submerged in a medium of the following composition in Gm./liter: glucose 10, soybean meal 15, distillers' solubles 5, and sodium chloride

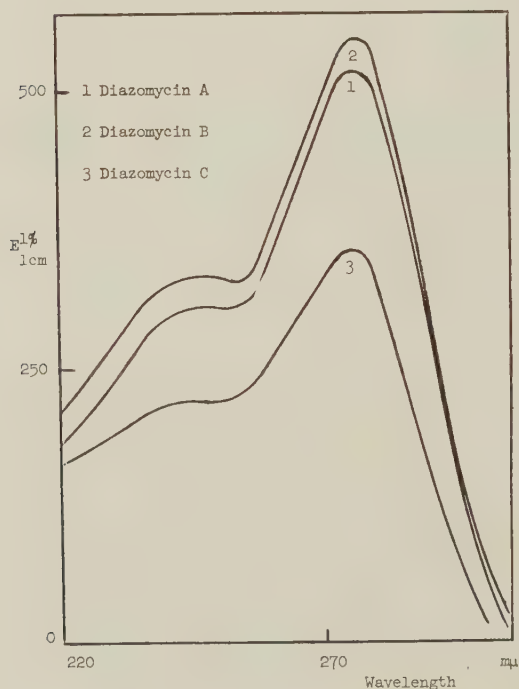


FIG. 5. The ultraviolet spectra of diazomycins.

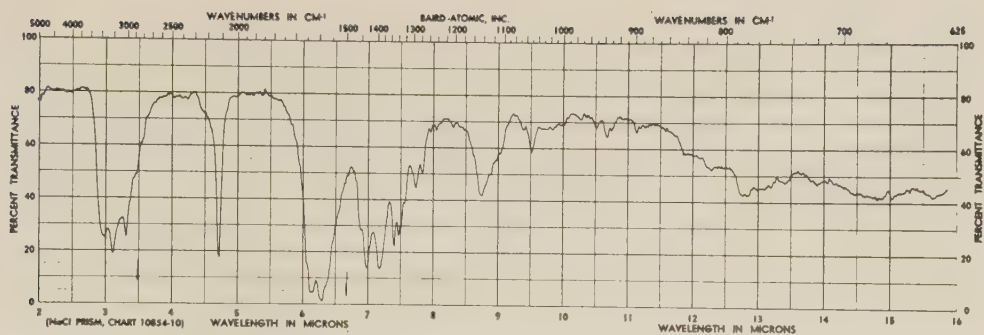


FIG. 6. Infrared spectrum of diazomycin A.

2. The ratio of the various diazomycins produced depends on time. The fermentations are usually harvested between 45 to 65 hours. After filtration through a bed of Hyflo Supercel, the broth is concentrated under reduced pressure (30 to 35 C.) to about 5 to 10 per cent of the original volume. The concentrate is then added with vigorous stirring to 10 volumes of methanol and the mixture filtered. The filtrate is concentrated at 30 C. to remove most of the methanol. The concentrate is then brought to 5 per cent solids and passed through a column of Darco (4 to 8 Gm./Gm. of solids). The sample is followed with water and then with 5 to 10 per cent acetone. Fractions are assayed both by microbiological and spectrophotometric methods. The active fractions are combined, concentrated, and freeze dried.

Alternatively the concentrate is diluted to 2 per cent solids and passed through a column of Dowex-1 acetate (5 to 10 ml. resin per Gm. of solid). The "D" fraction appears in the effluent unless the column is overloaded. After the sample, the column is washed with water and then eluted with 1 per cent phosphate buffer pH 7.5. The elution is complete when the optical density difference which rises to a peak falls off to a negligible value. The fractions are combined based on the paper chromatographic evidence and freeze dried.

Diazomycin A. The partition column was set up with either Celite or silica gel with the system 2.5 per cent phosphate buffer (pH 7.0) and 4:1 mixture of *n*-butanol and isopropyl alcohol. The sample from the above procedure was added to the column and developed with the mobile phase. The active fractions were combined, shaken with water, and the aqueous layer concentrated and freeze dried.

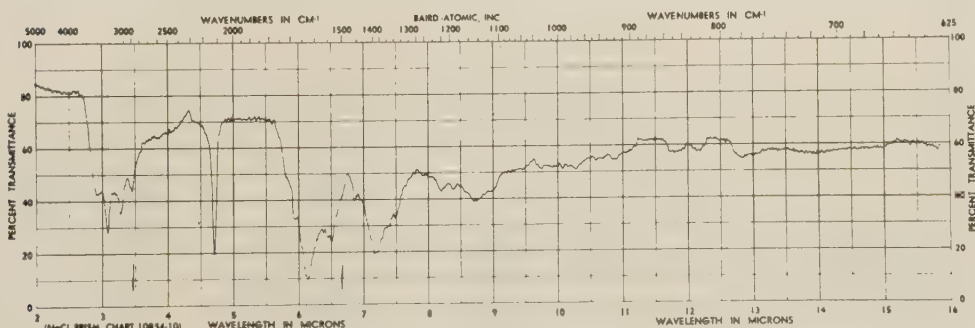


FIG. 7. Infrared spectrum of diazomycin B.

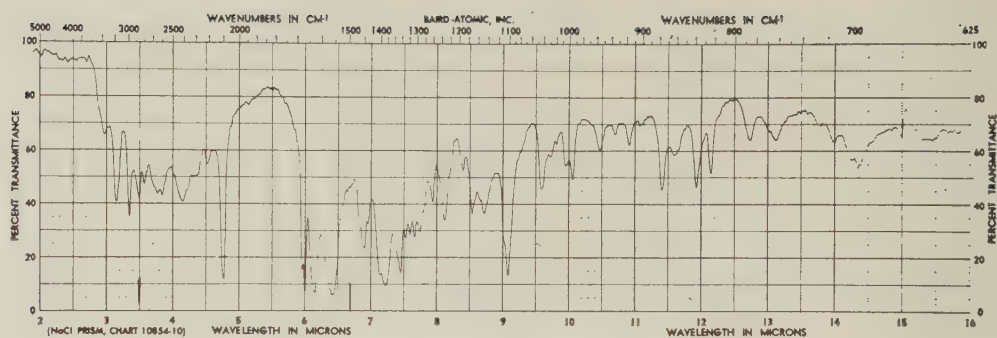


FIG. 8. Infrared spectrum of diazomycin C.

A solution of this product was passed through Amberlite IR-120 in lithium cycle. The product after being freeze dried was taken up in ethanol, filtered, and concentrated in presence of pyridine. A pale yellow thick gel separated out which was filtered and washed with pyridine. After drying over sulfuric acid the substance is dissolved in ethanol and concentrated. An almost transparent pale yellow gel-like product separated. This was filtered, washed with ethanol, and dried. This product was very hygroscopic and, when left exposed to air, absorbed moisture, and soon turned into a crystalline mass. The crystals appear as clusters of pale yellow needles, $E_{1\text{cm}}^{1\%}$ 530 at 275 $m\mu$. Analysis. Found: C, 40.31; H, 5.24; N, 17.10. Found on anhydrous sample: C, 44.34; H, 5.08; N, 17.22.

Diazomycin B. The crude material from the ion exchange column was subjected to countercurrent distribution (100 transfers) between phenol and water. Diazomycins A and B accumulated in tubes 40 to 60 as was judged by optical density at 275 $m\mu$ and by activity against *B. subtilis*.

The "A-B" mixture was recovered and added to Dowex-1 acetate (25-50 ml./Gm. of sample) and eluted with 0.3 to 0.5 per cent phosphate buffer. The eluate fractions were followed by optical density, microbiological activity, and quantitative ninhydrin color reaction. Diazomycin B gives an intense purple color and the color peak coincides with the optical density. The appropriate fractions are combined and freeze dried. The product was extracted with methanol several times and the combined extract concentrated to 25 per cent of its volume and refiltered to remove any more salts which separated out. Further concentration and filtration gave pale yellow crystals of diazomycin B which were recrystallized twice from aqueous methanol. The product separated out as sulfur-yellow rectangular plates, $E_{1\text{cm}}^{1\%}$ 550 at 275 $m\mu$. Analysis. Found: C, 43.15; H, 5.59; N, 19.22.

TABLE I
Properties of Diazomycins A, B, and C

Property	Diazomycin A	Diazomycin B	Diazomycin C
1. Ultraviolet extinction coefficients			
275 $m\mu$	520	550	340
245 $m\mu$	315	340	210
2. Ninhydrin color reaction	Light grey-blue	Intense purple	Light grey-blue
3. Solubility in methanol	Readily soluble	Slightly soluble	Readily soluble
4. <i>B. subtilis</i> activity units/mg.	100	10,000	<1
5. R_f value (80% isopropanol)	0.6-0.7	0.2-0.3	0.8-1.0

Diazomycin C. This component accumulated in the 100 tube distribution between phenol and water in tubes 20 and 30. The material was recovered and freeze dried. The partition chromatography procedure for the purification was the same as used in the case of diazomycin A. The active material was recovered by extraction with water and freeze drying. This product was shaken with ethanol, filtered, the filtrate concentrated, and set aside after adding a small amount of acetone. The crystalline product which separated out was recrystallized from ethanol-acetone mixture. Diazomycin C separates out as cream colored needles, $E_{1\text{cm}}^{1\%}$ 340 at 275 m μ . Analysis. Found: C, 46.03; H, 5.93; N, 23.82.

Fraction "D." Active fractions from the effluent of the ion exchange column were combined and freeze dried. The product was subjected to a 100 tube distribution between phenol and water. The activity accumulated between tubes 30 to 40. The compound was recovered and the procedure repeated on it. The product from this was shaken with ethanol and filtered to remove some soluble impurities. Extraction of the residue with methanol and concentration gave a cream colored crystalline solid which was recrystallized from aqueous methanol whereby it separated out as very pale yellow fine needles, $E_{1\text{cm}}^{1\%}$ 670 at 275 m μ . Comparison of the crystalline material with an authentic sample of 6-diazo 5-oxo norleucine by infrared spectra, paper chromatography, and microbiological activity showed that they are identical.

SUMMARY

From the culture filtrates of the actinomycete, *Streptomyces ambofaciens* are isolated three antitumor agents designated as diazomycins A, B, and C, together with 6-diazo 5-oxo norleucine. These compounds exhibit properties characteristic of aliphatic diazo ketones. They show marked inhibitory activity against sarcoma 180 and adenocarcinoma 755, and moderate activity against leukemia 1210.

ACKNOWLEDGMENT

The authors are grateful to Mr. S. C. Beesch and Mr. E. K. Hamilton for the pilot plant batches and to Dr. R. L. Wagner for physical measurements. Appreciation is expressed to Mr. J. L. Davenport for continued interest and encouragement in the work.

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Streptonigrin, An Antitumor Substance

I. Isolation and Characterization

KOPPAKA V. RAO AND WALTER P. CULLEN

Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

In our Microbiology Department an actinomycete culture was isolated, the broth filtrates of which when tested by Dr. Christine Reilly at Sloan-Kettering Institute, as well as in our own laboratories, showed a high degree of antitumor activity. The activity was especially pronounced against adenocarcinoma 755 in mice and human tumor type HS #1 grown in rats. The broths also showed considerable antibiotic activity against both gram-positive and gram-negative bacteria. The culture was characterized as *Streptomyces flocculus* and the description and characteristics will appear in a separate publication. The isolation and characterization of the active principle is the subject matter of this communication.

The fermentations are carried out by the submerged culture technique in small stirred jars as well as in medium sized tanks (100 gallons). The culture grows well in several of the commonly available complex natural media. The progress of the fermentation is followed by activity against *Bacillus subtilis* and *Micrococcus pyogenes* var. *aureus*. The production of the active principle reaches a maximum at about 50 to 60 hours and there is no appreciable change up to about 90 hours.

Preliminary experiments showed that the antitumor activity parallels the antibiotic activity. After some degree of purification, optical density measurements could be used for assay purposes.

For recovery of the active principle, the filtered broth is extracted with a solvent such as *n*-butanol or ethyl acetate in the pH range 3.0 to 5.0. Almost all the activity is extracted by the solvent. Concentration of the extract, passage of the active material into a neutral buffer followed by re-extraction into ethyl acetate at pH 4.0, and re-concentration gives the crude active substance in the form of a coffee-brown powder.

Paper chromatography and preliminary distribution studies showed that the activity is due to a major component, which accounted for about 80 per cent of the total activity. The remainder is divided between one or two minor components. Purification is carried out conveniently by countercurrent distribution in the system ethyl acetate—3 per cent phosphate buffer, pH 7.5. The distribution is carried out in a 100 tube automatic countercurrent distribution apparatus. The progress of the distribution was followed by optical density measurements at 370 m μ as well as activity against *M. pyogenes* var. *aureus*. The compound is recovered from the appropriate fractions by extraction into a solvent such as ethyl acetate or chloroform at pH 4.0 and crystallized first from ethyl acetate and then from acetone or dioxane. The major active component is named streptonigrin because of its dark brown color.

It was noted that the distribution coefficient of streptonigrin varies with the concentration. However, the product after the distribution and crystallization could

FIG. 1. The counter-current distribution of streptonigrin.

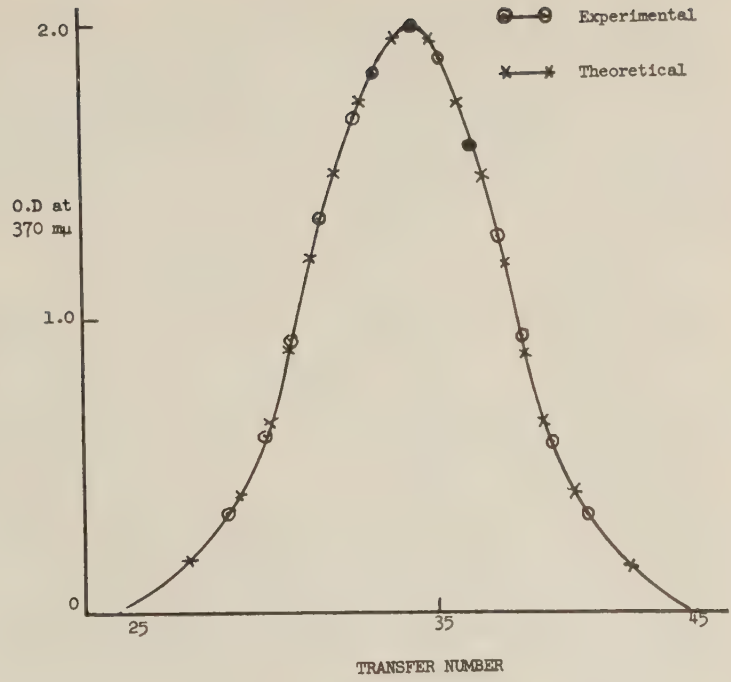
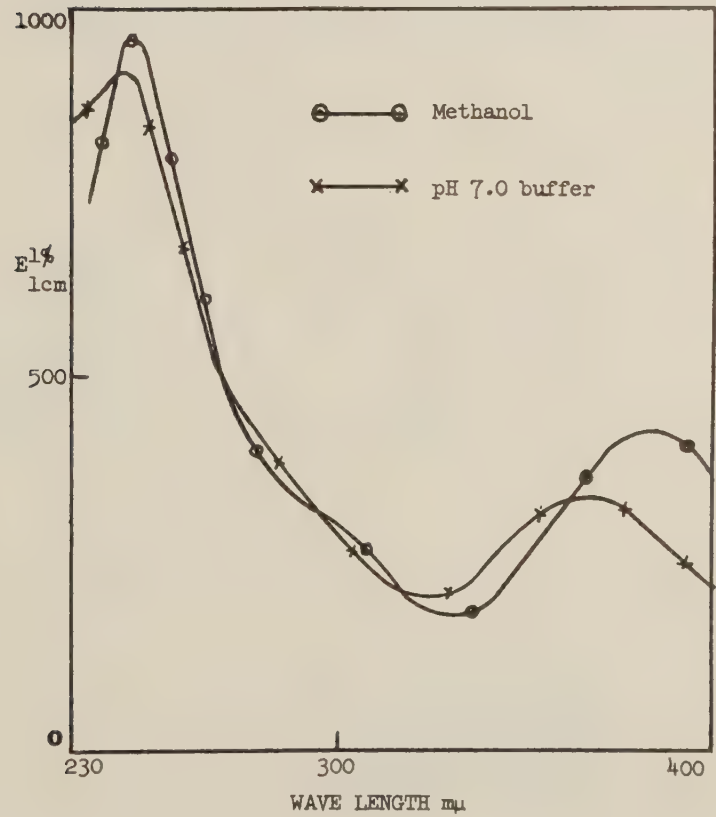


FIG. 2. The ultraviolet spectrum of streptonigrin.



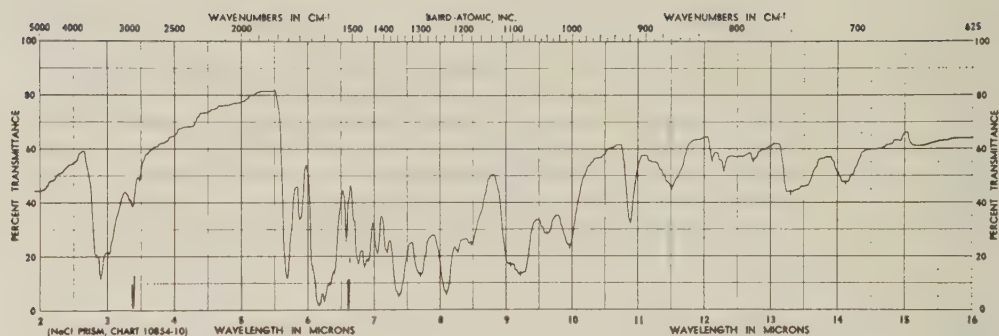


FIG. 3. The infrared spectrum of streptonigrin.

be shown to be homogeneous by a countercurrent distribution in a more dilute solution, as shown in figure 1.

Streptonigrin crystallizes as coffee-brown to almost black, long rectangular plates depending on the solvent from which it is crystallized. It decomposes at about 275 C. It is slightly soluble in water, lower alcohols, ethyl acetate, or chloroform. It dissolves more readily in dioxane, pyridine, or dimethyl formamide or aqueous sodium bicarbonate although with gradual decomposition in this latter case. Streptonigrin analyzes as follows: Found: C, 58.66; H, 4.93; N, 11.01. Calculated for $C_{24}H_{20}O_8N_4$: C, 58.53; H, 4.09; N, 11.38. Calculated for $C_{24}H_{22}O_8N_4$: C, 58.30; H, 4.48; N, 11.33. It behaves as a weak acid and gives a pK_a value of 6.2-6.4 in 1:1 aqueous dioxane solutions. The ultraviolet and infrared spectra of streptonigrin are shown in figures 2 and 3. Some of its characteristic color reactions are shown in table I.

Streptonigrin is stable at room temperature for 24 hours and at 100 C. for 15

TABLE I
Color Reactions of Streptonigrin

1. Alcoholic ferric chloride	dark greenish brown
2. Concentrated sulfuric acid	deep yellow
3. Aqueous sodium hydroxide	dark greenish brown changing to red with evolution of ammonia
4. Sodium hydrosulfite	changes to yellow and reverts to brown on shaking with air
5. Bromine in chloroform	color changes to yellow.
6. 2,4-Dinitrophenylhydrazine	orange red precipitate

TABLE II
Antibiotic Activity of Streptonigrin

Microorganism	Minimum inhibitory concentration, $\mu g./ml.$
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	0.39
<i>Bacillus subtilis</i>	<0.09
<i>Mycobacterium tuberculosis</i> 607	0.19
<i>Salmonella typhosa</i>	3.12
<i>Klebsiella pneumoniae</i>	1.56
<i>Pseudomonas aeruginosa</i>	6.25

minutes in the pH range 2.0 to 7.0. Beyond pH 8.0, substantial decomposition takes place. Also solutions are photosensitive especially at pH 7.0 or higher.

The antibiotic activity of streptonigrin is shown briefly in table II.

Crystalline streptonigrin shows a high degree of activity against adenocarcinoma 755 in mice and against HS #1 in rats and only a moderate activity against sarcoma 180 and leukemia 1210.

SUMMARY

Streptonigrin is an antibiotic substance with antitumor activity isolated from the broth filtrates of *Streptomyces flocculus*. It is a dark brown crystalline solid with an empirical formula of $C_{24}H_{20.22}O_8N_4$. It behaves as a weak acid with quinonoid properties. It shows broad-spectrum antibiotic activity in vitro and significant antitumor activity in experimental animals.

ACKNOWLEDGMENT

The authors are grateful to Mr. S. C. Beesch and Mr. E. K. Hamilton for the pilot plant batches, to Dr. R. L. Wagner for physical measurements, and to Dr. A. R. English for the antibacterial spectrum.

Excretion and Tissue Distribution of Actinobolin

JAMES G. CAPPUCCINO, HERBERT F. OETTGEN, M. LOIS MURPHY,
H. CHRISTINE REILLY, AND JOSEPH H. BURCHENAL

Divisions of Experimental and Clinical Chemotherapy, Sloan-Kettering Institute for Cancer Research, Department of Medicine, Memorial and James Ewing Hospitals, and the Sloan-Kettering Division of Cornell University Medical College, New York, N. Y.

Actinobolin was first shown to be active against sarcoma 180 in the mouse by Reilly,¹ and in other rodent tumor systems by Sugiura and Reilly.² Burchenal et al³ have demonstrated activity against a wide spectrum of transplanted mouse leukemias. They also have shown that strains of leukemia L1210 that have developed resistance to amethopterin or mercaptopurine or both, or to glyoxal *bis* (guanyldihydrazone) are not resistant to actinobolin. This suggests a mechanism of action differing from that of the previously known antileukemic agents. Thus, this new antibiotic appeared to be potentially interesting for the chemotherapy of neoplastic diseases in man.

Eighteen children⁴ and 13 adults⁵ have been treated with actinobolin sulfate by our group. The drug, prepared as a 1 to 2 solution in 5 per cent dextrose in water, was administered intravenously in most of these cases. In these patients the maximum daily dose ranged from 20 to 400 mg./Kg., with total doses of 100 to 5700 mg./Kg./course of the treatment. Immediate signs of toxicity were anorexia, nausea, and vomiting, fever, decrease in blood pressure, occasionally with shock, and increase in blood urea nitrogen. Symptoms of delayed toxicity were mouth ulcers, rash, erythema, desquamation, alopecia, leukopenia, and thrombocytopenia.

The following effects were observed: There was regularly a decrease in white counts of patients with acute leukemia, accompanied by signs of toxicity. When administration of the drug was discontinued, white counts rose again. There was no marrow response in these cases. In a patient with breast cancer and hypercalcemia, there was a significant decrease in serum and urinary calcium. Two other patients with carcinoma of the kidney and carcinoma of the testis and hypercalcemia did not show such a response.⁶

In addition, in some patients actinobolin had been administered subcutaneously, the maximum daily dose being 150 mg./Kg., with total doses up to 2200 mg./Kg./course. There were painful areas at sites of injection and side effects comparable to those after intravenous administration at the higher dose level.

Two children with acute leukemia have been treated orally. At a dose level of 286 mg./Kg. (total dose 7 Gm.) daily, one child developed loose stools. There was no response of the disease, nor were there any side effects during five weeks of treatment. The other child died before having received a comparable dose.

These studies were supported by research grants CY 3215 and 3192 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, by grants from the American Cancer Society and by the Damon Runyon Memorial fund for cancer research and the Black-Stevenson fund. This work was supported by Contract No. SA-43-ph-2445, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health.

TABLE I

Urinary Excretion of Actinobolin in Man after Intravenous Administration,
Dose: 100 mg./Kg. (1.36 Gm.)

Time, hours	Urine volume, ml.	Actinobolin		Blood serum, γ/ml.
		γ/ml.	Total mg.	
0*	15	<40	0.0	<40
1	36	1133	40.8	60
2	15	6400	96.0	
4†	36	6960	250.6	60
6	45	3200	144.0	
8	75	740	55.5	<40
24	100	285	28.5	<40
Total amount excreted		615.4		

* Actinobolin infusion started.

† Actinobolin infusion finished.

It appeared that actinobolin showed some therapeutic effect, especially in children with acute leukemia. On the other hand, the drug produced so many undesirable side effects after intravenous, and even after subcutaneous administration, that its usefulness seemed to be seriously limited. That toxicity did not occur after oral administration may have been due to poor absorption from the gastrointestinal tract. Pittillo et al⁷ have demonstrated in rats that the urinary excretion of actinobolin is much lower after oral than after parenteral administration. In the present study, attempts have been made to determine whether this is also true in patients. In addition, we have tried to ascertain whether the low urinary excretion in animals after oral administration was due merely to poor absorption or to destruction of the drug in the gastrointestinal tract, and if it was possible to enhance the absorption. Finally, the tissue distribution of actinobolin and the influence of different tissues on the destruction of the drug were studied.

MATERIALS AND METHODS

In all experiments, actinobolin sulfate has been used.

Preliminary in vitro studies showed that there was no loss of activity after storage at 4 C. for 24 hours, when actinobolin was dissolved in human serum, heparinized blood, heparinized plasma, oxalated blood, oxalated plasma or urine. Accordingly, the blood and urine samples obtained from the patients were frozen, or, if kept at 4 C., assayed within 24 hours. Dose schedules, being different in each case, will be described later. Clinical studies were carried out in 3 children and 1 adult patient. In animal experiments, C58F₁ (F₁ hybrids of the Bagg albino female × C58 male cross) mice (20 to 22 Gm.) and Wistar albino female rats (200 to 300 Gm.) were used. The concentrations of actinobolin in urine, blood, and tissue were assayed by a paper disc-nutrient agar plate method, with *Sarcina lutea* as the test organism.⁷ Standard solutions of actinobolin sulfate and all experimental samples were prepared in 0.1 M phosphate buffer at pH 7.8. Standard curves were prepared in the range of 20 to 100 γ/ml.

CLINICAL STUDIES

In 2 patients, the urinary excretion after intravenous administration was followed.

TABLE II

Urinary Excretion of Actinobolin in Man after Oral Administration
Dose: 200 mg./Kg. (3 Gm.)

Time, hours*	Urine		
	Volume, ml.	Actinobolin	
		γ /ml.	Total mg.
0	28.0	<40	0.00
2	56.0	58.0	3.24
4	38.0	480.0	18.20
6	32.0	760.0	24.30
8	50.0	555.0	27.70
24	235.0	140.0	32.80
Total amount excreted	106.20		

* Actinobolin 750 mg. given orally at hours 0, 1, 2, and 3.

Table I shows the results in one of these patients (E. R., 4 year old girl. Diagnosis: Rhabdomyosarcoma of left labium maius with metastases to bones and cervical nodes). She received 100 mg./Kg. (1360 mg.) of actinobolin in 500 ml. 5 per cent dextrose in water over a period of four hours. No untoward side effects were observed. It is evident that the urinary excretion of actinobolin began immediately after the infusion had been started, reaching its peak concentration at four hours (end of the infusion) and decreasing rapidly thereafter. Blood levels of 60 γ /ml. were found during the four hour infusion period. Four hours after termination of the infusion, no actinobolin (<40 γ /ml.) could be detected in the blood. The total amount excreted in the urine after 24 hours was 45 per cent of the dose given in this patient, and 50 per cent after 20 hours in the other patient (R. G., 16 year old boy with chronic granulocytic leukemia). Urinary excretion of actinobolin after oral administration of 117 mg./Kg. (1750 mg.) and 200 mg./Kg. (3000 mg.) was determined in patient E. R. Table II shows the results in the study at the greater dose level. In comparison with results after intravenous administration, urinary excretion was delayed; the peak of excretion was reached six to eight hours after oral administration had been started. The total amount excreted after 24 hours was 3.5 per cent of the dose given, that is, less than one tenth of the excretion after intravenous administration. The results of the study at the lower dose were qualitatively similar, but quantitatively were proportional to the amount of drug given. No toxicity was observed in either experiment.

In an attempt to compensate for the poor absorption from the gastrointestinal tract by giving higher doses, a patient (M. H., 10 year old boy, osteogenic sarcoma of left femur with lung metastases) was given 400 mg./Kg. (13 Gm.) divided into 11 hourly doses. The patient developed diarrhea, however, and the urinary excretion of actinobolin within 24 hours was only 2.3 per cent of the dose given. No further excretion occurred thereafter.

Finally, a patient (G. K., 4 years old, with rhabdomyosarcoma of right scapular region with lung metastases) was given 200 mg./Kg. (3 Gm.) once every morning orally on five consecutive days. Table III shows the amounts excreted daily in the urine. It is evident that there is some increase in excretion from the first to the fourth day. During the fourth day, the patient developed diarrhea. Urinary excre-

TABLE III

Urinary Excretion of Actinobolin in Man after Oral Administration on 5 Consecutive Days
Dose: 200 mg./Kg. (3 Gm.)/day

Day	Volume, ml.	Urine		
		Actinobolin		Per cent excreted
		γ /ml.	Total mg.	
0	236.0	<40	0.0	
1	286.0	265.0	65.7	2.1
2	288.0	400.0	115.2	3.8
3	195.6	855.0	167.0	5.6
4	210.0	815.0	179.3	6.0
5	166.7	765.0	127.5	4.3
6	220.0	<40	0.0	

tion decreased on the fifth day, when the last dose was given. No drug was excreted on the sixth day.

Because, after oral administration, urinary excretion was only one tenth or less than after intravenous injection, it appeared that the absorption of actinobolin from the gastrointestinal tract of patients probably was poor. There was a slight increase of urinary excretion when drug administration was continued over several days, possibly as a result of absorption also from the lower parts of the gastrointestinal tract. The urinary concentration, however, was still much lower when compared with intravenous administration. The occurrence of diarrhea limited the dose which could be given orally.

ANIMAL EXPERIMENTS

Because the clinical studies indicated that the rate of urinary excretion of actinobolin was low when the drug was administered orally, animal experiments were undertaken in an attempt to enhance the absorption of actinobolin from the gastrointestinal tract. A preliminary experiment was carried out to establish the urinary excretion pattern of rats receiving actinobolin by various routes of administration. Pairs of rats were given a dose of 1500 mg./Kg. by the intraperitoneal, subcutaneous, and oral routes. Because little variation in results was obtained with individual rats in each pair, results for each treatment were averaged.

Data in table IV indicate that the amount excreted in the urine in 24 hours

TABLE IV

Urinary Excretion of Actinobolin in Rats on Different Routes of Administration
Dose: 1500 mg./Kg. in 10 Per Cent Solution

Hours after administration	Excretion after administration, mg.		
	Intraperitoneal	Subcutaneous	Oral
0	0.0	0.0	0.0
5	87.0	54.0	4.5
24	24.8	32.3	9.7
Total dose excreted, mg.	111.8	86.5	14.2
Total dose given, mg.	241.0	250.0	259.0
Per cent of total dose excreted	46.4	34.6	5.5

TABLE V

Urinary Excretion of Actinobolin in Rats 24 Hours after Oral Administration in Different Vehicles
Dose: 1500 mg./Kg. in 20 Per Cent Solution

Vehicle	Water	Water and glucosamine	Peanut oil	Propylene glycol
Total dose given, mg.	238.0	232.0	359.0	352.0
Excreted after 24 hours, mg.	8.5	13.0	37.6	16.3
Per cent of total dose excreted	3.6	5.1	10.6	4.6

following intraperitoneal, subcutaneous, and oral administration was 46.4, 34.6, or 5.5 per cent, respectively, of the dose which was administered. These data are similar to those obtained with patients and tend to confirm that little absorption occurs when actinobolin is administered orally. In later experiments with rats, not shown in this table, an average of 20 per cent of the given dose was found in the intestinal contents 24 hours after oral administration, as against 0.4 per cent after intravenous administration.

In an attempt to enhance absorption of actinobolin from the gastrointestinal tract, addition of glucosamine, suspension of the drug in peanut oil and in propylene glycol, and the effect of *pH* were studied.

The results obtained with glucosamine, peanut oil, and propylene glycol are shown in table V. During the first 24 hours after administration, rats given glucosamine plus actinobolin excreted 5.1 per cent; those given a propylene glycol suspension of the drug, 4.6 per cent; and those given actinobolin in peanut oil suspension, 10.6 per cent of the administered dose. The control rats, given 1500 mg./Kg. of actinobolin in water, excreted 3.6 per cent of the total dose.

In another experiment, rats were treated orally with actinobolin in 0.1 *M* buffer solutions of sodium acetate at *pH* 5.9 and 6.9, in phosphate buffer *pH* 7.8, and in Tris buffer *pH* 9.0. When actinobolin was prepared as a 10 per cent solution with these buffer preparations, the final *pH* values obtained were 5.0, 6.0, 7.0, and 8.0, respectively. Data showing the effect of different *pH* values on urinary excretion after oral administration are in table VI. Slightly greater quantities of the drug were recovered in the urine after the administration of solutions at *pH* 6.0, 7.0, and 8.0, than after administration at *pH* 5.0. In no case, however, did the excretion approach the quantities found after parenteral administration.

The tissue distribution of actinobolin was studied in mice and rats. Groups of 5

TABLE VI

Effect of pH on Urinary Excretion of Actinobolin in Rats 24 Hours after Oral Administration
Dose: 1500 mg./Kg. in 10 Per Cent Solution

Buffer	Sodium acetate <i>pH</i> 5.0	Sodium acetate <i>pH</i> 6.0	Phosphate <i>pH</i> 7.0	Tris <i>pH</i> 8.0
Total dose given, mg.	342.0	339.0	368.0	368.0
Excreted after 24 hours	11.2	27.3	19.3	23.1
Per cent excreted	3.2	8.0	5.0	6.2

TABLE VII

Distribution of Actinobolin in Mouse Tissues on Different Routes of Administration
Dose: 800 mg./Kg.

Tissue	γ actinobolin/Gm. tissue on administration				
	Intravenous*	Intraperitoneal*	Subcutaneous*	Oral†	Oral‡
Brain	0	0	0	0	0
Liver	>1000	840	690	320	<400
Spleen	340	520	250	180	200
Stomach	530	200	220	>1000	<400
Small intestine	560	340	<400	>1000	110
Large intestine	580	<400	<400	>1000	>1000

* Mice sacrificed 15 minutes after injection.

† Mice sacrificed 1 hour after injection.

‡ Mice sacrificed 5 hours after injection.

mice were given 800 mg./Kg. of actinobolin by the intravenous, intraperitoneal, subcutaneous, and oral routes. The mice treated parenterally were sacrificed 15 minutes after drug administration; those given the drug orally were sacrificed after one and five hours. Brain, liver, spleen, kidney, stomach, and large and small intestines from the 5 mice were pooled. The contents of the gastrointestinal tract were not removed. Each tissue was ground in a Virtis Tissue Homogenizer and diluted 1:5 with 0.1 M phosphate buffer, pH 7.8. Table VII shows the distribution of actinobolin in mouse tissues. After parenteral administration, the highest concentration of drug was found in liver tissue: the values for spleen, stomach, and intestine did not differ significantly. After oral administration, the highest concentrations of actinobolin were found in the intestinal tract. Actinobolin was present in the large intestine one hour after administration. After five hours, the large intestine contained far more drug than the other tissues. At both these time intervals, the values for liver and spleen were lower than those 15 minutes after parenteral injection. Actinobolin could not be detected in brain tissue in any of the groups. Homogenates of all tissues, except those of kidney, from nontreated control animals showed no inhibition of growth of assay organism. Normal kidney homogenates produced such large zones of inhibition that it was impossible to determine, with any degree of certainty, the concentration of actinobolin in the kidneys of treated mice.

TABLE VIII

Distribution of Actinobolin in Rat Tissues at Different Times after Subcutaneous Administration
Dose: 1500 mg./Kg.

Tissue	γ actinobolin/Gm. tissue				
	1/2*	1	3	9	24
Brain	0	0	0	0	0
Liver	210	400	310	<200	0
Stomach	310	<200	0	0	0
Small intestine	<200	430	340	<200	0
Large intestine	0	0	0	<200	520

* Time of sacrifice after injection, in hours.

TABLE IX

Distribution of Actinobolin in Rat Tissues at Different Times after Oral Administration
Dose: 1500 mg./Kg.

Tissue	γ actinobolin/Gm. tissue				
	1/2*	1	3	9	24
Brain	0	0	0	0	0
Liver	0	0	0	<200	<200
Stomach	36,000	3250	3150	910	0
Small intestine	0	2500	18,500	<200	0
Large intestine	0	0	1225	12,400	4150

* Time of sacrifice after injection, in hours.

Rats were given 1500 mg./Kg. of actinobolin subcutaneously or orally and sacrificed at different times after administration, as indicated in tables VIII and IX. The organs were processed as in the mouse experiment. With untreated rats, both kidney and spleen caused such a high degree of inhibition of *Sarcina lutea* that results obtained with these tissues from actinobolin-treated animals could not be properly evaluated. The data obtained were consistent with those in the mouse experiment. Again, no actinobolin was detectable in the brain. Also, drug concentration in the liver after parenteral administration was higher than after oral administration. The drug appeared in the large intestine three hours after oral administration and was still detectable there after 24 hours. Small amounts of actinobolin were found in the gastrointestinal tract after parenteral administration (table VIII). The pattern of passage into the large intestine was similar to that observed after oral treatment.

Attempts were made to determine whether or not actinobolin might be destroyed enzymatically by mouse and rat tissues. Experiments were conducted in 0.1 *M* phosphate buffer, pH 7.8, at 37 C. As a control, actinobolin sulfate was dissolved in buffer in a concentration of 100 γ /ml. and incubated at 37 C. After various intervals of time, aliquots were removed and assayed. As may be seen in table X, the antibiotic was destroyed rapidly under these conditions. Solutions of actinobolin to which homogenates of brain, liver, spleen, kidney, stomach, small and large intestines had been added showed the same degree and rate of destruction.

DISCUSSION

Because toxic side effects make the parenteral use of actinobolin hazardous, particularly in adults, the desirability of oral administration of the drug was in-

TABLE X

Destruction of Actinobolin in Vitro at 37 C.

Incubation time, hours	Actinobolin, mg./ml.
0	100
1	90
6	60
12	<20

vestigated. It might be possible to maintain constant blood levels—therapeutically active and yet not high enough to produce toxicity—by giving frequent, regularly repeated doses orally. Actinobolin, however, has been shown to be poorly absorbed from the gastrointestinal tract after oral administration in patients as well as in mice and rats. Attempts to improve the absorption in rats by using different vehicles and by buffering the drug at different *pH* values have so far failed. The results of the animal experiments suggest that the poor absorption of actinobolin is not due to rapid destruction of the drug, since a considerable amount passed through to the large intestine. It was surprising to find actinobolin in the large intestine 24 hours after administration, although in vitro the drug, dissolved in 0.1 *M* phosphate buffer at *pH* 7.8, was completely destroyed after 24 hours at 37 C. It is possible that this type of buffer system influences the stability of the antibiotic. In vitro studies did not show any difference in the rapidity of destruction when actinobolin was incubated with different mouse and rat tissues.

Finally, it is of interest that in mice and rats actinobolin was not detectable in the brain after intravenous, subcutaneous, or intraperitoneal injection. In this respect, actinobolin resembles methotrexate^{8,9} and 6-mercaptopurine.¹⁰

SUMMARY

The urinary excretion of actinobolin, in patients and rats, has been found to be much lower after oral than after parenteral administration. Similar differences have been obtained in studies of tissue distribution in mice and rats. Attempts to improve the gastrointestinal absorption by administering the drug in various vehicles and at different *pH* values have not been successful.

ACKNOWLEDGMENTS

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The In Vivo Antitumor Activity of Streptovitacins A, B, C₂, and D

JOHN S. EVANS, JOHN CERU, AND GORDON D. MENGEL

The Upjohn Co., Kalamazoo, Mich.

The isolation, characterization, and biological activity of streptovitamin A have been described.¹⁻⁴ The papergram identification of five members of the streptovitamin family and the relative potency of streptovitamins A and B against *Saccharomyces pastorianus* and *Trichomonas vaginalis* have been reported by Sokolski et al.⁵ He found that A and B were equally effective against *S. pastorianus*, whereas A was 10 times more effective than B against *T. vaginalis*.

This paper presents a comparison of the in vivo antitumor activity of crystalline streptovitamins A, B, C₂, and D and cycloheximide.

METHODS

Three tumor systems were used for assay purposes: the ascitic form of Ehrlich carcinoma and the solid forms of sarcoma 180 and Walker adenocarcinoma 256. The methods used for carrying the tumors were the same as previously described.⁴

The total packed cell volume was used as the basis for measuring the activity of streptovitamins A and B in ascitic mice. The procedure used was the same as that described by Sassenrath et al.⁶ with the following changes: The number of animals per group was a minimum of 10, 0.2 ml. was used for injection, and injections were made daily for seven days.

The Walker assay was conducted by implanting the tumor subcutaneously into groups of 10 or more Sprague-Dawley male rats weighing 65 ± 5 Gm. The drug was given intraperitoneally once daily for 10 days starting 20 hours after implanting the tumor. The tumors were measured in two diameters 24 hours after the last injection. The average tumor diameter was used for the calculation of results.

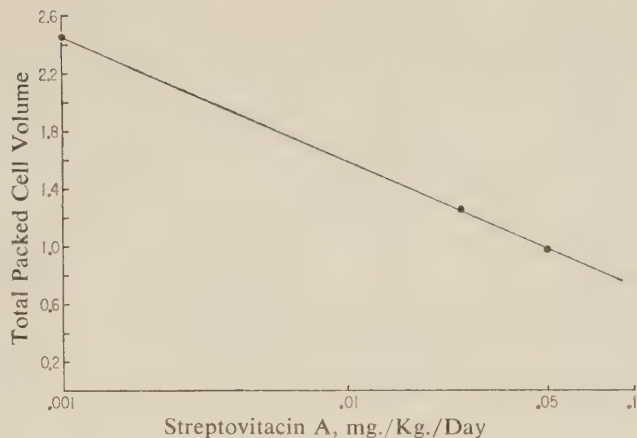
The sarcoma 180 assay was conducted by implanting the tumor into groups of 10 mice weighing 18 to 22 Gm. The drug was given intraperitoneally for seven days starting 20 hours after implanting the tumor. The tumors were measured in two diameters or removed and weighed 20 hours after the last injection. The average tumor diameter or weight was used for the calculation of results.

One per cent carboxymethyl cellulose was used as the solvent for the streptovitamins. Cycloheximide, which is chemically related to the streptovitamins, has been studied in both carboxymethyl cellulose and in an alcohol-cottonseed oil vehicle. The alcohol-oil vehicle (1 volume of ethanol plus 19 volumes of cottonseed oil) was chosen for routine studies for cycloheximide because of the variable stability of the compound in aqueous media under our conditions.

RESULTS

Dosage response curves (figs. 1-3) for streptovitamin A were determined using three tumor systems. Straight line dose response curves were obtained when the average tumor diameter, total packed cell volume, or per cent tumor inhibition was plotted against the logarithm of the dosage. Spacing of the dosage was dependent

FIG. 1. The effect of streptovitamin A on Ehrlich carcinoma (ascites) is shown.

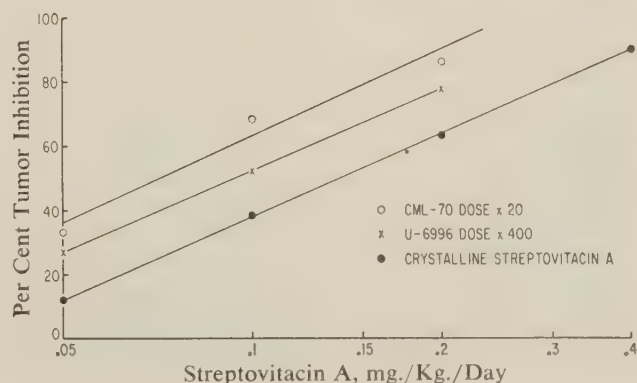


upon the activity and toxicity of the compound. The ability of one of the test methods to assay potency of different samples of the same material, varying in purity, is shown in figure 2. The relative activity of the streptovitamins and cycloheximide was determined using two of the tumor systems. It is evident (table I) that streptovitamin A is about 70 to 100 times more active than B or C₂ when tested in any of the three in vivo test methods. Streptovitamins A and D have the same order of activity against Walker 256 (tables I, II). Both are more active than cycloheximide.

Smith et al⁷ have studied the effect of the same series of compounds in tissue culture against the KB strain of human epidermoid carcinoma cells. The in vitro cytotoxic activity of streptovitamins A, B, C₂, and D and cycloheximide reported by Smith et al agrees well with the relative in vivo activity of the same compounds against the Walker tumor system.

Streptovitamin A had the same order of activity on a body weight basis against the rat tumor, Walker 256, and the mouse tumor, sarcoma 180. Approximately 4 to 7 per cent of the acute LD₅₀ dose of streptovitamin A produced 50 per cent inhibition of either Walker 256 or sarcoma 180. Cycloheximide, on the other hand, was more active against Walker 256 than against sarcoma 180. Four per cent of the acute LD₅₀ dose of cycloheximide produced 50 per cent inhibition of the Walker tumor, whereas 25 per cent of the acute LD₅₀ dose was required to produce a similar

FIG. 2. The effect of streptovitamin A on Walker 256 is illustrated.



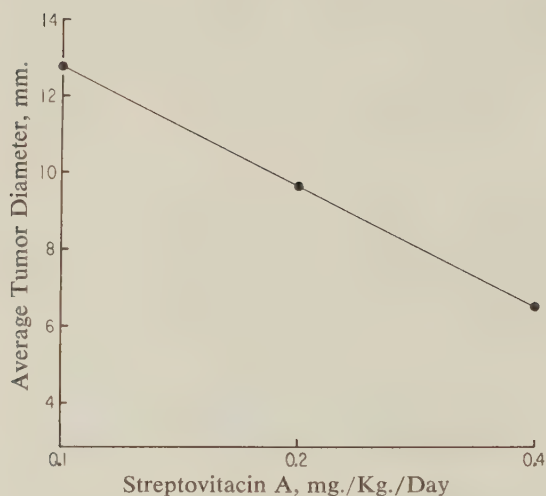


FIG. 3. The effect of streptovitamin A on sarcoma 180 is shown.

inhibition of the sarcoma 180. Our results with cycloheximide were similar to those reported by other investigators.⁸

Mefferd and Loefer⁹ showed that cycloheximide sharply inhibits the endogenous respiration of *Tetrahymena pyriformis* S as well as its utilization of fructose, galactose, glucose, mannose, maltose, acetate, α -ketoglutarate, lactate, maleate, pyruvate, glutamic acid, and tryptophane. The authors concluded that the site of action of cycloheximide seems to be common to many reactions and may be of a nonspecific nature.

Kerridge,¹⁰ using *Saccharomyces mandschuricus*, showed that concentrations of cycloheximide which completely inhibited protein synthesis did not affect respiration or fermentation. At the same concentrations, nucleic acid synthesis was markedly inhibited initially and ceased after a short interval. Further investigation showed that the nucleic acid formed under these conditions consisted only of ribonucleic acid, to the exclusion of deoxyribonucleic acid.

Smith et al⁷ have shown that streptovitamins A, B, C₂, and D and cycloheximide

TABLE I
Antitumor Activity of Streptovitamins and Cycloheximide

Compound	Test method	Amount, mg./Kg./day, to give 50% tumor inhibition
Streptovitamin A	Walker assay	0.15–0.20
Streptovitamin B	Walker assay	20.0
Streptovitamin C ₂	Walker assay	>2.0
Streptovitamin D	Walker assay	0.15–0.20
Cycloheximide	Walker assay	0.4
Streptovitamin A	Sarcoma 180	0.2–0.4
Streptovitamin B	Sarcoma 180	>20.0
Streptovitamin C ₂	Sarcoma 180	>20.0
Cycloheximide	Sarcoma 180	25.0
Streptovitamin A	Ehrlich carcinoma (total packed cell volume)	0.03
Streptovitamin B	Ehrlich carcinoma (total packed cell volume)	2.0

TABLE II

Comparative Assays of Streptovitacins A and D and Cycloheximide Using Walker 256

Compound	Dose, mg./Kg./day	No. survivors	Tumor diameter, mm.	Standard deviation	Standard error	σ_D^*
None (tumor control)		20†	35.33	12.87	2.88	
Streptovitamin A	0.05	19	26.00	9.88	2.27	3.66‡
	0.10	20	18.70	7.70	1.72	3.35
	0.20	20	13.73	7.94	1.78	3.38
	0.40	19	4.55	5.35	1.23	3.13
Streptovitamin D	0.20	10	12.75	5.37	1.70	3.34
	0.40	10	8.10	2.99	0.95	3.03
	0.20	10	26.00	10.53	3.33	4.40‡
Cycloheximide	0.40	10	17.60	4.33	1.37	3.19
	0.80	10	5.60	5.10	1.61	3.30

* σ_D Standard error of difference between means of test group and controls.

† Twenty animals were used for tumor control and for each streptovitamin A level. Ten were used for each level of streptovitamin D and cycloheximide.

‡ Probability of observed deviation from control is 0.05. Probability for all other groups is <0.01.

inhibit protein synthesis in KB cells. It is not known whether the streptovitacins exhibit other metabolic effects, such as those already noted for cycloheximide.

CONCLUSION

Streptovitacins A and D are equivalent in activity when tested against Walker 256 and are more active than cycloheximide.

Streptovitamin A was found to be 70 to 100 times more active than B and C₂ when tested against Walker 256, sarcoma 180, or Ehrlich carcinoma (ascites).

The in vivo activity of the streptovitacins agreed well with the in vitro cytotoxic activity reported against KB cells.

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Experiences with Cyclophosphamide in Treatment of Childhood Tumors

HENRY G. CRAMBLETT*

Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City, Iowa

The present study, which was initiated in December of 1958, was undertaken to determine the efficacy of cyclophosphamide[†] in the therapy of childhood tumors as well as to gather information pertaining to its toxicity. This report will summarize our observations on the first 10 patients to whom this drug was given.

PATIENT MATERIAL

Patients to whom cyclophosphamide was administered were ones who had previously received conventional therapy for their cancers with one exception (patient 10). At the onset of therapy with cyclophosphamide, all patients had clinical and laboratory evidence of active malignant disease.

The patients were between the ages of 4 and 12 years. The cancers for which they were treated included Hodgkin's disease (3), acute leukemia (2), lymphoma (2), rhabdomyosarcoma (1), and Wilm's tumor (2) (table I).

CLINICAL PHARMACOLOGY

Cyclophosphamide was supplied in 100 and 200 mg. vials containing sodium chloride. When an appropriate amount of doubly distilled water was added, a solution of cyclophosphamide in physiological saline resulted.

The drug was administered intravenously during an interval of two to three minutes within 30 minutes after being put into solution. If the contents of a vial were not completely used, the remaining solution was discarded. Initially, each patient was given a small test dose (25 to 50 mg.) intravenously in the effort to detect an idiosyncratic reaction to the drug before larger amounts were administered. The drug was then given every day intravenously in doses of 50 to 100 mg. Cyclophosphamide was continued intravenously until a leukopenia developed or until a total dose of at least 35 mg./Kg. had been given. There were two exceptions to this (patients 6 and 7). These were patients with leukemia who were leukopenic prior to onset of therapy with cyclophosphamide and to whom smaller doses were given.

TOXICITY

Six of the 10 patients developed a leukopenia in the peripheral blood during the course of intravenous therapy with cyclophosphamide. The differential counts of

* Present address: Department of Pediatrics, The Bowman Gray School of Medicine, Winston Salem, N. C.

† The trade name of Mead Johnson & Co. for cyclophosphamide is Cytosan.

TABLE I
Summary of Cases

Pa- tient	Age, yr.	Diagnosis	Previous treatment	Result*	Cyclophosphamide, total dose/mg./Kg./ total days	Oral, av. daily dose, mg.	Result*	Toxicity
1	9	Hodgkin's lymphoma	1-59 Irradiation	± <1 mo.	1 Gm./40 mg./Kg./16d	50	0	Transient leukopenia
2	8	Hodgkin's lymphoma	5-58 Irradiation	+7 mo.	1.5 Gm./70 mg./Kg./17d	50	+4½ mo.	Transient leukopenia
3	12	Hodgkin's lymphoma	12-58 Irradiation	+2 mo.				
			7-53 Surgery					
			8-53 Surgery	+24 mo.	1 Gm./35 mg./Kg./10d	75	+3 mo.	None
			11-53 Surgery					
			11-55 Irradiation	+18 mo.				
4	9	Lymphoma anaplastic	5-57 Surgery	+20 mo.				
			8-58 Irradiation	+3 mo.	(A) 0.8 Gm./35 mg./Kg./8d	100	+5 mo.	Transient leukopenia
			11-58 Actinomycin D	+1 mo.				
			12-58 Irradiation, nitrogen mustard	+1 mo.	(B) 1 Gm./40 mg./Kg./10d		+1 mo.	
5	5	Lymphoma	1-59 Irradiation	0				
			9-56 Surgery & nitrogen mustard	+23 mo.	0.85 Gm./50 mg./Kg./10d	50	±1½ mo.	Alopecia
			8-58 6-Mercaptopurine	0				
			9-58 Irradiation	+2 mo.				
			1-59 Actinomycin D	±2 mo.				
6	4	Acute myelomonocytic leukemia	12-58 Irradiation	+1 mo.	0.275 Gm./20 mg./Kg./7d	50	±3 mo.	None
			1-59 Myleran	0				
			1-59 Prednisone	0				
7	4	Acute lymphocytic leukemia	5-58 6-Mercaptopurine	+3 mo.	0.4 Gm./30 mg./Kg./5d	50	±1 mo.	None
			8-58 Amethopterin	+4 mo.				
			12-58 Prednisone	+3 mo.				
			2-59 6-Mercaptopurine riboside	+1 mo.				
8	9	Rhabdomyosarcoma	7-54 Surgery	+43 mo.	(A) 0.5 Gm./25 mg./Kg./8d	100	+3 mo.	Transient leukopenia
			8-54 Surgery					
			2-58	+3 mo.				
			5-58 Surgery	+3 mo.	(B) 0.5 Gm./25 mg./Kg./5d		+1 mo.	
			8-58	0				
			10-58					
			11-58					
			11-58 Actinomycin D	+2 mo.				
9	4	Wilm's tumor	1-58 Nephrectomy	+6 mo.	1 Gm./55 mg./Kg./18d	50	0	Transient leukopenia
			1-58 Irradiation	+3 mo.				
			7-58 Irradiation	+3 mo.				
			10-58 Irradiation	+5 mo.				
10	9	Wilm's tumor	Simultaneous surgery and irradiation		0.9 Gm./40 mg./Kg./9d	—	0	Transient leukopenia†

*0 = no effect; ± = partial remission; + = complete remission.

† Result of simultaneous irradiation and cyclophosphamide.

leukocytes remained normal—there was not a selective depression of the polymorphonuclear cells. The platelet counts did not change significantly during therapy.

The leukopenia did not become evident until after 5 to 14 days of intravenous therapy. The leukocyte count usually became normal within 7 to 10 days after interruption of therapy. In 2 patients, the leukocyte count became depressed during oral therapy with cyclophosphamide.

None of the children experienced nausea, vomiting, headache, or inflammation at the site of injection. In one patient alopecia was observed.

RESULTS

The results of therapy and duration of remissions are summarized in table I.

Two (patients 2 and 3) of the 3 patients with Hodgkin's disease had complete remissions for three and four and one half month periods. The third patient (patient 1) had been previously treated with irradiation with equivocal beneficial effect of short duration, and received no further benefit from cyclophosphamide.

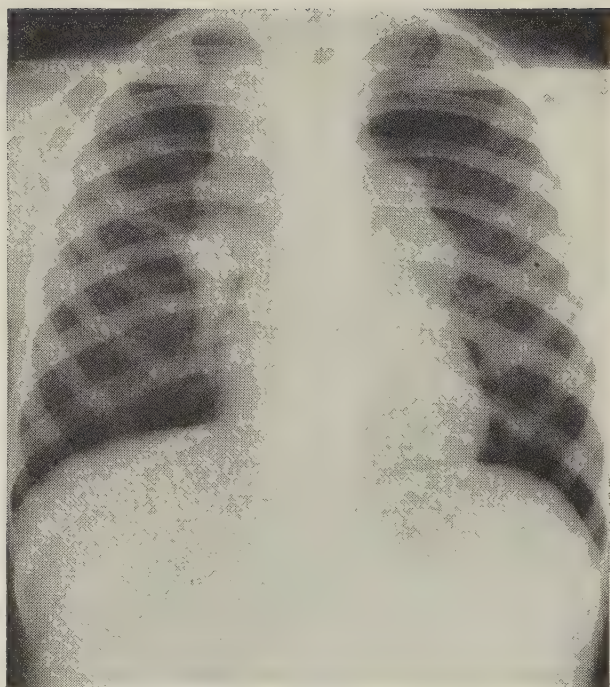


FIG. 1. Mediastinal lymphadenopathy prior to therapy in patient 4.

One (patient 4) of 2 patients with anaplastic lymphoma responded dramatically to therapy with cyclophosphamide. This patient had proptosis of the left eye and marked cervical and mediastinal lymphadenopathy. All of these abnormal findings regressed with therapy. The regression of the mediastinal lymphadenopathy is shown

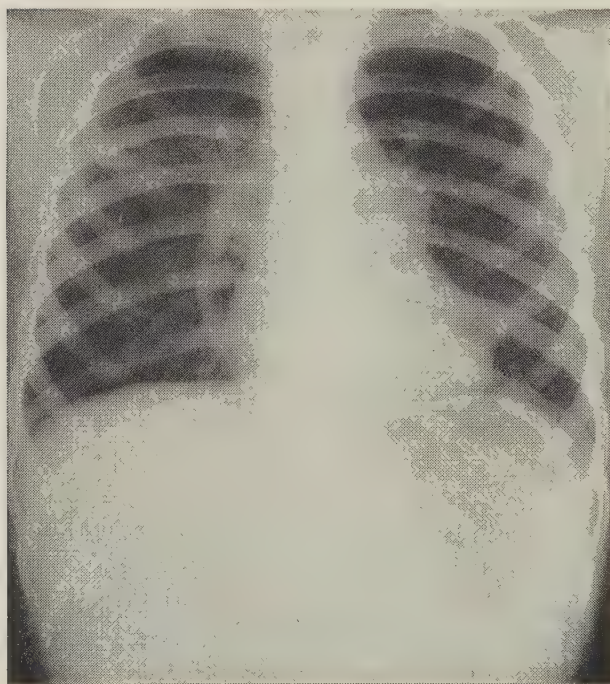


FIG. 2. Demonstration of regression of mediastinal lymphadenopathy in patient 4 following cyclophosphamide therapy.

in figures 1 and 2. The beneficial effects persisted for five months. The patient relapsed while on oral therapy and was given a second course of cyclophosphamide intravenously. This produced a second remission which persisted for one month.

Two patients with leukemia (patients 6 and 7) had partial remissions during therapy with cyclophosphamide. In one patient (patient 7), there was relief of bone pain. In the other patient (patient 6), transfusions of blood were not necessary during a three month period; previously he had required a transfusion every other week.

One of the most striking effects of therapy with cyclophosphamide was in a patient with rhabdomyosarcoma (patient 8). At the beginning of therapy the patient was moribund. She was febrile, cachectic, and had marked inguinal lymphadenopathy and abdominal ascitic fluid. After therapy, for the first time in six months, the patient became ambulatory and gained weight. Three months later while on oral cyclophosphamide, the patient experienced a relapse. She was given another course of intravenous therapy and obtained beneficial effects for an additional month.

Two patients with Wilm's tumors were treated. In one patient (patient 9), cyclophosphamide was given for treatment of a solitary pulmonary metastatic nodule that had become evident following nephrectomy and irradiation. There was no measurable effect. In the other patient (patient 10), cyclophosphamide was given at the time of surgery and subsequently in association with irradiation to prevent metastatic spread of the tumor. Nonetheless, three months later, several pulmonary metastatic nodules were noted on chest roentgenograms.

SUMMARY

Nine patients with various types of childhood cancer who had relapsed following conventional therapy were treated with cyclophosphamide. In a tenth patient, the drug was used initially in conjunction with surgery and nephrectomy for therapy of a Wilm's tumor. Complete remissions were observed in 4 and partial remissions in 3. There were no detectable beneficial effects in two patients with Wilm's tumors. Toxicity included transient leukopenia in 6 patients and alopecia in 1. The results of this study indicate that cyclophosphamide is a useful drug in the therapy of certain cancers.

Further Observation of the Clinical Effectiveness of Mitomycin C, an Antitumor Antibiotic

YAEMON SHIRAHA, KATSUJI SAKAI, KENICHI HASHIMA, AND
HIROO FUKUSUMI

Department of Surgery, Osaka City University Medical School, Osaka, Japan

Since the isolation of fraction C of mitomycin, produced by a strain of soil fungi, *Streptomyces caespitosus*, the antitumor activity of mitomycin C has been studied both experimentally and clinically in many institutions in Japan and widely all over the world, particularly in the United States.²² It has already been ascertained that mitomycin C has a wide range of antitumor activity against animal cancers,¹⁸ and clinical evaluation is at present in progress on various types of cancer in human beings.

Shiraha et al¹⁷ and Teranaka²¹ reported preliminary trials of this new tumor-inhibiting antibiotic in 82 cases in the field of general surgery at the previous annual meeting of the Symposium on Antibiotics and emphasized its bright prospects in antitumor chemotherapy. Since the preliminary report, we have accumulated 112 additional cases of cancer treated with mitomycin C (until the end of July, 1959), and we, furthermore, have made long-term observations of the patients, including previously reported cases. Accordingly, we are going to report here the results of clinical evaluation of mitomycin C in 194 patients with cancer. In the future, we also want to survey and analyze 663 cases treated with the same antibiotic during the past few years in other institutions in Japan.

DOSAGE SCHEDULE AND METHOD OF ADMINISTRATION

In principle, 2 mg. of mitomycin C is administered intravenously once a day. We continued this dosage until the leukocyte count of the peripheral blood was reduced to 2000–2500 cells/cu. mm. Moreover, we either administered the antibiotic intra-arterially, through a polyethylene tubing indwelt in the supply artery of the malignant lesion on indicated cases, or injected the drug directly into the tumor tissue itself and the adjacent area (table I).

CLASSIFICATION OF PATIENTS

Clinical diagnosis of the patients treated with mitomycin C was as follows: stomach cancer, 95; breast cancer, 16; rectum cancer, 14; cancer of the maxilla or mandible, 13; seminoma, 7; bronchiogenic cancer, 6; primary cancer of the liver, 6; uterine cancer, 6; cancer of the tongue, 3; 2 cases each of cancer of the intestine, pancreas, larynx, parotid gland, and bronchiogenic origin; and 1 case of urinary bladder cancer. In addition, there were: malignant lymphoma, 5; sarcoma of the maxilla, 4; 2 cases each of fibrosarcoma and mediastinal tumor; and 1 case each of sarcoma of the omentum, Hodgkin's disease, orbital tumor, and chronic myelogenous leukemia.

From 117 patients of our series, we were able to get specimens for histological

TABLE I
Classification of Routes of Administration of Mitomycin C

	Route			
	Intravenous	Intra-arterial	Topical	Total
Number of cases	143	44	7	194

classification of the cancer, as shown in table II; adenocarcinoma accounted for 67.5 per cent.

One hundred fifty-one of 194 cases had far-advanced cancer without any hope of radical surgery; they were placed on chemotherapy, and the remaining 43 were also placed on prophylactic mitomycin C to prevent either metastasis or recurrence after complete or incomplete radical surgery for malignant lesions.

CLINICAL EFFECTS OF MITOMYCIN C THERAPY

Subjective improvement felt by the patients after mitomycin C administration is, of course, one of the criteria to evaluate the effectiveness of the drug. It has been manifested as increased appetite, less discomfort, relief of pain, and mitigation of cough and sputum, particularly in the early stages after starting mitomycin C therapy. After mitomycin C therapy, Shiba et al¹¹⁻¹³ observed increased appetite in 25 and less discomfort in 20 of 80 patients; Tasaki et al²⁰ reported subjective improvement in 33.3 per cent of 47 patients with cancer. However, subjective improvement is not always a reliable criterion by which to evaluate the effectiveness of an antitumor substance. For this reason, we should use objective criteria in evaluations. This is an important problem in antitumor chemotherapy, and it is not definitely solved yet. For the present, we have recognized a few signs, shown in table III, as objective improvement after mitomycin C administration for cancer.

There were 38 patients in whom malignant tumors were reduced remarkably in size after mitomycin C administration, including complete disappearance of the tumor in 1 patient. However, there were many cases in which the regressed tumor increased in size again after discontinuance of the drug. A few patients with carcinomatous peritonitis, who had been suffering from oliguria and had not responded to any diuretics, showed remarked decrease of ascites and, consequently,

TABLE II
Histological Classification of the Cancers Treated with Mitomycin C

Classification	Number of cases	Per cent
Adenocarcinoma	79	67.5
Undifferentiated carcinoma	13	11.1
Squamous cell cancer	9	7.7
Mucoid carcinoma	5	4.3
Basal cell cancer	1	0.9
Fibrosarcoma	5	4.3
Reticulosarcoma	3	2.6
Round cell sarcoma	2	1.7

TABLE III

Objective Clinical Effects of Mitomycin C in 194 Cases

Classification of clinical signs	Number of cases out of		
	Intravenously administered, 143 patients	Intra-arterially administered, 44 patients	Topically used, 7 patients
Regression of tumor	28	10	
Reduction of ascites and increase of urine volume	8	5	
Gain of body weight	5	3	
Improvement of passage	4	3	
Improved chewing	4	0	
Necrosis of tumor	1		3

immediate increase of urine volume after mitomycin C therapy. Reduction of ascites resulted in disappearance of abdominal distention, relief of breathlessness, and improved appetite.

Gain of body weight is assumed to have resulted from improved appetite. There were 7 patients who had had stenosis caused by cancer at the pylorus or cardia of the stomach and showed alleviation of intractable nausea and vomiting owing to regression of the tumor size. Patients with chewing difficulty all had cancer of either the maxilla or the mandible and showed immediate improvement of their difficulty up to normal chewing activity.

Mitomycin C was infiltrated topically into either the tumor tissue itself or adjacent tissue when the tumor had been ulcerated superficially and discharged secretion. Many of the patients responded with reduction of the discharge, leaving an odorless necrotizing substance. Shiba et al¹³ treated 80 cases of cancer with 1 to 4 mg. of intravenous mitomycin C per day; they observed regression of tumor size in 21, softening of the tumors in 6, reduction of ascites in 8, decrease of edema in 4, and improvement of passage disturbance in 6. Similar clinical effects were achieved by Shimada et al.¹⁴ Tasaki et al²⁰ observed improvement of general condition in 10, tumor regression in 5, and reduction to disappearance of ascites in 5 of 50 patients given mitomycin C therapy; they pointed out accelerated healing of cancerous ulcers, reduction of edema, and decreased jaundice. However, these clinical responses of the patients to mitomycin C were almost all temporary, getting worse after discontinuance of the drug. Mitomycin C was unable to provide a complete cure of far-advanced cancer patients and assured only some life-retaining effects. Hibino et al⁴ treated a series of leukemia cases with mitomycin C and noticed that 6 of 8 patients with chronic myelogenous leukemia showed clinical responses manifested as improved general condition, reduction of splenomegaly, normalization of the blood picture, and clinical remission within a period of more than four months. A case of primary polycythemia responded excellently to mitomycin C, as seen by both blood picture and splenomegaly, resulting in a satisfactory clinical remission. But there were no responses to mitomycin C in 3 cases of acute myelogenous leukemia and in 1 case of myeloma.

Ashitaka et al¹ placed their gynecological patients on mitomycin C therapy with carcinostatic response on squamous cell carcinoma of the cervix or the vulva and

cancer of the ovary. They administered the drug through both systemic and topical routes, such as by instillation into the peritoneal cavity, local infiltrative injection, and local application in a preparation of mitomycin C ointment or vaginal suppository; they emphasized the value of its topical use because of reduced rate of leukopenia.

Four cases of primary pulmonary cancer given mitomycin C by Hattori et al³ showed reduction of fever, cough, sputum, and chest pain, with improved general condition but without any roentgenographic evidence of tumor size reduction.

Of 13 cases of skin cancer treated with mitomycin C by Higuchi and Uematsu, 1 case of cancerous ulceration in the frontal region disappeared completely after intravenous administration of 2 mg. of the drug per day for 27 days.

LONG-TERM OBSERVATIONS

Many of the patients with advanced cancer were discharged from the hospital under improved general and local conditions. We have made a follow-up study of the patients six months or more after completion of mitomycin C therapy. There were 3 survivors of the patients with far-advanced cancer, as shown in table IV. There were many patients who had been discharged from the hospital in an improved condition and had been doing well six months or more, and relapsed thereafter to be placed again on antitumor chemotherapy. Two case reports from this series were presented in a previous paper.¹⁷ A third case report follows.

CASE REPORT

A 74 year old Japanese man with cancer of the urinary bladder gave a history of sudden onset of hematuria at the age of 69 years, which subsided on medical treatment, relapsing several times since. However, this hematuria became uncontrollable by medical treatment in April, 1958, being complicated with severe pollakiuria and anemia. The patient was referred to the Department of Surgery when cystoscopy and roentgenograph revealed a cancer larger than a hen's egg at the left side posterior wall of the urinary bladder.

At admission, the patient was severely starved, showing anemic conjunctivae. Laboratory examinations revealed the red blood cell count to be 3,010,000 cells/cu. mm.; hemoglobin, 46 per cent; white blood cell count, 8400 cells/cu. mm.; and platelet count, 160,000/cu. mm., with normal bleeding and coagulation times and bloody urine mixed with huge cancer cells.

On Aug. 14, 1958, a polyethylene tubing was inserted into the common iliac artery through one of the branches of the right femoral artery, its intra-arterial stump being fixed at the

TABLE IV
Three Survivals of the Far-Advanced Cancer Patients Treated with Mitomycin C

Route of administration	Patient Age, yr. Sex	Clinical diagnosis	Surgery	Total dose of mitomycin C, mg.	Surviving period, months
Intravenous	T.K. 51 Male	Cancer of the jaw	None	69	20
	R.Y. 56 Male	Stomach cancer	Gastro- jeunos- tomy	36	16
Intra-arterial	Y.I. 74 Male	Cancer of the urinary bladder	None	66	10

TABLE V

Long-Term Observation of the Patients Placed on Prophylactic Mitomycin C after Radical Surgery

Clinical diagnosis	Relapsed or died	Number of cases			
		Without evidence of recurrence			Total
		20 months or more	10 months or more	6 months or more	
Stomach cancer	3	1	7	3	11
Breast cancer	0	4	6	1	11
Seminoma	0			2	2

bifurcating site of the abdominal aorta. The patient was given 2 mg. mitomycin C per day through the intra-arterial tubing, with 66 mg. in total dose over a period of 37 days. Hematuria began to decrease in grade around 10 days, with complete disappearance 20 days after institution of chemotherapy. During a course of mitomycin C therapy, blood transfusion was added every four days, with 500 ml. of the citrated blood in total volume. The prechemotherapeutic anemia was remarkably improved, revealing red blood cell count 4,120,000 cells/cu. mm. and hemoglobin content 79 per cent 14 days after commencement of antibiotic therapy.

The patient has gained body weight and has been doing well 10 months since discharge from the hospital, without surgery.

PROPHYLACTIC USE

As mentioned previously, we also studied 43 patients with cancer who had undergone either complete or incomplete radical surgery and been placed on prophylactic mitomycin C. Twenty-seven of 31 patients were followed six months or more after completion of antitumor chemoprophylaxis; their present status is classified in table V.

There were 3 patients with stomach cancer who developed relapse of the malignancy, despite prophylactic administration of mitomycin C after radical surgery; 1 of these patients died. However, the remaining 24 cases (stomach cancer, 11; breast cancer, 11; and seminoma, 2) have been quite free from any evidence of recurrence of cancer. In fact, our sampling of the patients is too small and the period of observation too short to evaluate long-term effectiveness of mitomycin C, so that the possibility of mitomycin C preventing recurrence of cancer after radical surgery is not definitely known at present. But it seems that prophylactic mitomycin

TABLE VI

Frequent Side Effects of Mitomycin C Therapy in Japan

Author	Total number of patients treated with mitomycin C	Number of cases of			
		Leukopenia	Bleeding tendency	Exanthemas	Anorexia
Shiba et al ¹¹⁻¹³	80	48	6		8
Shimada et al ¹⁴	58	25			2
Tasaki et al ²⁰	47	27	2		3
Tasaka et al ¹⁹	36	14	3		2
Okinaka et al ¹⁰	23	6	1		2
Fukushima et al ²	22	8	1	5	4
Higuchi and Uematsu ⁵	13	4			
Okawara et al ⁹	7	2			

TABLE VII

Leukopenia Three Weeks after Institution of Mitomycin C Therapy

Changes of leukocyte count in the peripheral blood	Route of administration			
	Intravenous		Intra-arterial	
	Number of cases	Per cent	Number of cases	Per cent
Reduced	124	87	27	61
Unchanged or increased	19	13	17	38

C is promising in the control of recurrence to some extent, and, in the future, we are going to accumulate data in order to evaluate real effectiveness of mitomycin C in this situation.

SIDE EFFECTS

We reported previously that mitomycin C caused few subjective side effects but, objectively, considerable leukopenia and, sometimes, slight bleeding tendency. In table VI are listed the most frequent side effects produced by mitomycin C in Japan.

Leukopenia. Almost all the investigators who used mitomycin C clinically observed leukopenia of the peripheral blood; its incidence in our studies is the highest (table VII), because we have administered mitomycin C as much as possible in the early stages of the antitumor chemotherapy, until the leukocyte count was reduced down to 2000–2500 cells/cu. mm. Table VII indicates a few interesting facts: that there have been some patients who did maintain normal leukocyte counts; that there rarely was hyperleukocytosis; and that the intra-arterial use of mitomycin C is preferable to intravenous use, because of reduced incidence of leukopenia.

Leukopenia produced by mitomycin C has been manifested mainly in myelogenic leukocytes, particularly neutrophils, with relative increase of lymphocytes in ratio. There was little change in rates of eosinophils and basophils. Moreover, no premature leukocytes were noticed in the peripheral blood.

Kosaki et al⁶ reported 2 patients with stomach cancer who had been placed on mitomycin C, 34 and 40 mg. total doses, respectively. Their leukocyte counts fell abruptly down to agranulocytosis after completion of the chemotherapy and they died.

Shimada et al¹⁴ have devised a modified dosage schedule for mitomycin C therapy, administering 4 to 6 mg. of the antibiotic as a single dose either once or twice a week. They emphasized advantages of their intermittent intravenous use of the drug because of reduced incidence of leukopenia and accelerated carcinostatic activity of mitomycin C. Nevertheless, we are still convinced of the superiority of the intra-arterial administration of the antitumor antibiotic in indicated cases, because this administration method gives selective distribution of the drug into the tumor tissue and reduced incidence of leukopenia, and it facilitates recovery.

Furthermore, it has been observed that such leukocytosis-promoting regimens as blood transfusion and combined use of cobalt-chlorophyllin or cystine all resulted

TABLE VIII

*Reduction of Leukocyte Count after Combined Use of Mitomycin C
with a Preparation of Essential Amino Acids*

Duration of mitomycin C administration (total dose)	Rates of reduction in leukocyte count	
	Mitomycin C alone, %	Combined use of mitomycin C with a preparation of essential amino acids, %
1 week (14 mg.)	10.7	8.7
2 weeks (28 mg.)	28.7	23.0
3 weeks (42 mg.)	43.6	37.8

in unsatisfactory recovery of the reduced leukocyte count, if the count was lower than 2000 cells/cu. mm. But some preparations, consisting of essential amino acids (Moriamin*), have prevented leukopenia produced by mitomycin C to some extent (table VIII).

Bleeding Tendency. In a previous paper,¹⁷ we touched very briefly on the bleeding tendency seen in some patients on mitomycin C therapy. In table IX is shown the correlation between increasing doses of mitomycin C and reduction rates of the platelet count and prothrombin time obtained in our own clinical trials. It is clear that systemic use of more than 40 mg. of mitomycin C has resulted in gradual decrease of these two factors, showing slight prolongation of bleeding time with a positive Rumpel-Leede's phenomenon. But no change of the coagulation time of the blood was noticed. These facts emphasize the importance of repeated platelet counts together with leukocyte counts during mitomycin C therapy.

Other Side Effects. Shiba et al¹³ reported disturbance of liver function during mitomycin C therapy, and Shimada et al¹⁴ reported some patients who had positive hematuria, albuminuria, and sometimes glucosuria after chemotherapy.

However, in our series there were no patients whose liver or renal functions were disturbed by mitomycin C therapy. Not infrequently anorexia has been experienced by the patients on mitomycin C, and Fukushima et al² reported nausea, vomiting,

TABLE IX

*Correlation Between Total Dose of Mitomycin C and Reduction Rates
of Platelet Count and Prothrombin Time*

Duration of administration and total dose of mitomycin C	Reduction rates of	
	Platelet count, %	Prothrombin time, %
1 week (14 mg.)	12.0	23.4
2 weeks (28 mg.)	23.2	31.5
3 weeks (42 mg.)	28.2	32.6

* An amino acid mixture solution in per cent as follows: 1-arginine hydrochloride, 0.8; 1-histidine hydrochloride, 0.40; 1-isoleucine, 0.55; 1-leucine, 1.23; 1-lysine hydrochloride, 2.23; 1-methionine, 0.71; 1-phenylalanine, 0.87; 1-threonine, 0.54; 1-tryptophane, 0.18; 1-valine, 0.61; glycine, 1.00. This solution was supplied by the Morishita Pharmaceutical Co., Osaka, Japan.

TABLE X

Side Effects of Mitomycin C Other than Leukopenia

Classification of side effects	Number of cases	
	Intravenously administered	Intra-arterially administered
Anorexia	15	1
Fever	2	9
Bleeding tendency	6	0
General fatigue	4	0
Violet stain of nail beds	3	0
Exanthemas	2	0

headache, and vertigo. A case of thrombophlebitis provoked by the drug was reported by Tasaki et al.²⁰

Side effects of mitomycin C other than leukopenia, as reported by other authors, are listed in the table X.

Anorexia was the most frequent side effect of mitomycin C therapy. The incidence of fever in the patients on intra-arterial mitomycin C was slightly higher than that with the intravenous route. The pyrogenic effect of mitomycin C has not been explained, and the fever is assumed to have resulted from the indwelling polyethylene tubing in the artery. In 3 cases violet discoloration of the nail beds was seen, which gradually faded over a long period of time after discontinuance of the drug without any evidence of harm to the patient's general condition. Exanthemas provoked by mitomycin C disappeared immediately after discontinuance of the drug and were also easily controlled by use of antihistaminics.

SUMMARY

At the Department of Surgery, Osaka City University Medical School, 194 patients with cancer were treated with mitomycin C during the past two years, from August, 1957, to July, 1959, and there have been accumulated 663 cancers of various origins treated with mitomycin C in Japan. The vast majority of the patients placed on mitomycin C had far-advanced cancer without any hope of cure by radical surgery, and they died within a period of two years despite vigorous antitumor chemotherapy. However, many of them responded satisfactorily clinically within a short period of time during or after mitomycin C therapy, which demonstrates the lifesaving effect of the drug on cancer.

It is also assumed that mitomycin C has prevented recurrence or metastasis of cancer after radical surgery, and this point will be pursued further in the future.

As a matter of fact, mitomycin C has provoked a few untoward side effects such as leukopenia and a bleeding tendency, which should be and can be prevented by careful use of the drug with reference to appropriate laboratory tests.

ACKNOWLEDGMENT

The authors wish to express their appreciation and thanks to Dr. S. Wakaki of

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The Present Status and Problems Involved in Human Sensitization to Antibiotics

Panel Discussion

Leo H. Crip, Moderator

Members of the Panel

Carl E. Arbesman
John M. Sheldon

Bernard B. Siegel
Howard I. Weinstein

Dr. Welch: Gentlemen, about two years ago I heard a panel discussion at Atlantic City, which impressed me tremendously. It was on a similar subject and the moderator was Dr. Leo Crip, who is Associate Professor of Medicine at the University of Pittsburgh. It was so well done that I asked Dr. Crip last year if he would come so that we might have a similar type of panel discussion for last year's symposium. Unfortunately, he was quite in demand and had to go to Paris at that time for another panel discussion. We were more fortunate this year and Dr. Leo Crip will moderate a panel for us on the present status and problems involved in sensitization to antibiotics. It is a great pleasure to have Dr. Crip with us.

Dr. Crip (Moderator): I believe it was Emerson who said that an institution is but the shadow of a man and these magnificent annual meetings reflect the organizing genius of your Chairman, Dr. Henry Welch. When he asked me if I would moderate a panel on the subject of allergy to antibiotics, he told me something about the audience and I realized that we were going to deal with a very critical audience and that the subject would be a difficult one to be dogmatic about. So I approached it with misgivings but I accepted anyway. I felt like "the prudent young lady of Kent/who knew what it meant/when men asked her to dine,/gave her cocktails and wine;/she knew what it meant—but she went."

Now, I hope you will be indulgent with us. We hope to cover certain phases of the subject of allergy to antibiotics, not all. We will talk about incidence; we will talk about various types of allergic reactions, their predictability and management, cross-antigenicity, and, perhaps, something about the role, the antigenic potential, of antibiotics in foods.

Now, moderating a panel is comparatively easy if you are smart enough to get high caliber panelists and this, I am sure, I have done and I would like to introduce them to you. Dr. John Sheldon, Professor of Medicine, University of Michigan School of Medicine, Dr. Carl Arbesman, Assistant Professor of Medicine, University of Buffalo School of Medicine, Dr. Bernard Siegel, Assistant in Medicine, New York State University College of Medicine, and Dr. Howard Weinstein, Director of Clinical Research, the Division of Antibiotics, Food and Drug Administration.

Now, with exception of Dr. Weinstein, we are mere clinicians, internists who are interested in clinical immunology.

We hope to have time left for questions from the floor and there are sheets of paper made available for this purpose. Actually, Dr. Welch should be up here because he has done a great deal of work with Dr. Weinstein on the incidence of allergy to antibiotics. Dr. Weinstein, would you care to tell us what is the incidence of allergy to penicillin?

Dr. Weinstein: The incidence of allergy to penicillin is very difficult to state categorically, which has been stated variously from 1 per cent all the way up to 16 per cent. I think a fair estimate would be that 5 to 6 per cent of all individuals are sensitive to some form of penicillin.

Dr. Criepp (Moderator): Dr. Weinstein, do you have any idea as to how many fatalities there are from penicillin yearly?

Dr. Weinstein: We made a survey of that a couple of years ago and the most common type of fatality due to penicillin sensitivity, of course, is the anaphylactoid shock. We uncovered 800 cases of anaphylactoid shock in a period of three years in about one third of all the hospitals that were canvassed throughout the country, one third of all the hospital beds. We assume from our statistics that there are approximately 3000 to 4000 cases of anaphylactoid shock due to penicillin and these carry an over-all mortality rate of approximately 10 per cent.

Dr. Criepp (Moderator): Many of these cases that you surveyed had not been reported in literature, am I right?

Dr. Weinstein: That is correct. None of them had been reported.

Dr. Criepp (Moderator): Now, what about the incidence of allergic reactions; remember that we are limiting ourselves now to a discussion of allergic reactions only and not to other reactions to antibiotics. Now then, what would be your guess as to the incidence of allergic reactions to broad-spectrum antibiotics?

Dr. Weinstein: The commonly used term "broad-spectrum antibiotics" applies to chloramphenicol and the tetracyclines. If we consider chloramphenicol, chlor-, oxy-, and tetracycline as a group, the over-all incidence of allergic manifestations to these drugs is very, very small, especially to the tetracyclines. We did a study about a year ago and it was very difficult, after canvassing practically the whole country and many allergists, to come up with many *bona fide* cases, documented cases of true allergy to tetracyclines. We finally managed to locate 7 cases; there are probably hundreds more, but over-all I would say that the incidence of allergy to the tetracyclines is very small.

The incidence of allergy to chloramphenicol is also small but here we are dealing with a different type of drug and if you consider the blood dyscrasias as a type of hypersensitivity, which is debatable, the percentage is also very small. I would not like to give a figure—I do not know of one.

Dr. Criepp (Moderator): Now we clinicians get terribly confused and mixed up by the different terms applied to the many antibiotics that are now available. Could you give us a quick run down of the main categories of broad-spectrum antibiotics, Dr. Weinstein?

Dr. Weinstein: Well, the broad-spectrum antibiotics, as I said before, include chloramphenicol or Chloromycetin and the tetracyclines: chlortetracycline, which is Aureomycin; oxytetracycline, which is Terramycin; and tetracycline itself. When tetracycline first was made available, it was manufactured by five companies under different names. Lederle put out their tetracycline under the trade name of Achromycin, Squibb put out theirs as Steclin, Upjohn had Panmycin, Bristol had Polycycline, and Pfizer had Tetra-

cyn. These five were not enough, and they decided to potentiate them. Upon potentiation, the names changed again and then we had about 15. So Achromycin potentiated with citric acid became Achromycin V and Steclin potentiated with hexametaphosphate became Sumycin, and Polycycline was discarded altogether by Bristol when they potentiated theirs and it became Tetrex. Upjohn did us a favor and kept Panmycin and Pfizer called theirs, when potentiated with glucosamine, Cosa-Tetracycl.

Dr. Crip (Moderator): In case those men who control the destinies of the pharmaceutical industry do not know, clinicians usually prescribe the antibiotic that was last recommended by the detail man, whose personality is such that he is irresistible. There is really a great deal of confusion. You told us at lunch today, Dr. Weinstein, about a sensitive patient who reacted severely to a usual dose of tetracycline. Would you mind repeating that?

Dr. Weinstein: Yes, we carried out a study about a year ago to determine what effect very small doses of the tetracyclines would have on these 7 patients that I mentioned before who were sensitive to therapeutic doses of tetracycline, namely, to the usual 250 mg. dose. We tested them with ascending doses, starting with 0.5, 1, 5, 10, and 25 mg. The reason for this is we were very interested in what harmful effects the use of chlortetracycline and oxytetracycline would have when used as preservative for fish in these people who were exquisitely sensitive to tetracycline, oxytetracycline, or chlortetracycline. Six of these 7 patients evidenced absolutely no signs of sensitivity to small doses up to 25 mg. of oxytetracycline or chlortetracycline, and these, mind you, were very sensitive to 250 mg. However, one individual reacted with all the manifestations that he reacted with to 250 mg. of tetracycline, namely, some wheezing, tearing of the eyes, nasal stuffiness, and some erythema, to 1 mg. of oxytetracycline, whereas it took 25 mg. of chlortetracycline to evince the same symptoms.

Dr. Crip (Moderator): When you say reaction you mean clinical reaction not merely a skin reaction.

Dr. Weinstein: No, he was not skin tested. It was a clinical reaction, which occurred within 10 minutes or thereabouts after administering the drug. This points up the fact that even though it is commonly accepted that if you are sensitive to one of the tetracyclines, you will probably be sensitive to all of them; there still is a quantitative difference, at least with some individuals, as to the amount of drug needed to elicit clinical symptoms of sensitivity.

Dr. Crip (Moderator): Would you say, Dr. Weinstein, that there is an increasing incidence of allergy to penicillin?

Dr. Weinstein: I would say there is an increasing incidence being reported. We see more, and we hear more of allergy to penicillin. This may be due to the fact that more is being used or that more people have been exposed to the drug, since both factors probably play an important role. I would say that probably there is a greater increase in the sensitivity, in the allergic manifestations to penicillin than formerly.

Dr. Crip (Moderator): Would you have any idea how much penicillin is used per year in this country?

Dr. Weinstein: Dr. Welch has those figures down. He uses them two or three times a day. I think it is in the neighborhood of 450 tons.

Dr. Criepp (Moderator): Dr. Sheldon, what would you say are the factors that lead to the development of any allergy to an antibiotic in a given person?

Dr. Sheldon: First, Dr. Criepp, I would think that the hereditary atopic background would play a very important role. Secondly, the amount and frequency of exposure to the antibiotic in question: the duration of its administration would probably also play an important role. Lastly, I would think that the mode of administration of the antibiotic would influence also the possibility of sensitizing the person in question.

Dr. Criepp (Moderator): Also, I suppose, when the antibiotic is given with an adjuvant, such as an oil.

Dr. Sheldon: That would also influence certain types of sensitivity.

Dr. Criepp (Moderator): Basically, then, you feel that the nature of the host is an important factor.

Dr. Sheldon: Very definitely.

Dr. Criepp (Moderator): I think it was Pasteur who said "the host is everything, the microbe is nothing." Let us move on. We hope to follow some set pattern in this discussion and in order to do this I would like to ask Dr. Arbesman to give us a breakdown of the classification of allergy to antibiotics. What different classes are there?

Dr. Arbesman: There are several different types of allergic responses to antibiotics. The most important one, of course, is the anaphylactic or anaphylactoid type. This is the immediate type of reaction in which after an injection or ingestion of a particular product the patient develops serious manifestations with collapse and fatality or develops wheezing, sneezing, difficulty in breathing, and has all the signs of a typical asthmatic with urticaria and so on. This is also an immediate type of allergic reaction.

Another type of allergic reaction is the delayed type or the serum sickness-like type reaction, which occurs anywhere from 7 to 10 days after the injection of penicillin. This occurs because there are antibodies developed in the patient's blood serum which reacts with antigen which is still circulating in the system. Antigen-antibody union occurs. This is evidenced by fever, which may be of low grade or may be up to 103 or 104 F. with urticaria, joint pains, and so on.

There is still another type of allergic response and that is the delayed type, namely, the contact dermatitis type of allergy. This is due to contact with the material, either in the form of ointments, powders as used by nurses or in the manufacture, eye drops, nose drops, or aerosols of one kind and another. This is an entirely different type of situation from the previous immediate type which has antibodies circulating in the serum. In contact dermatitis there are no positive reactions by the intradermal or scratch test but the patch test is positive. This is a very important differentiation to make.

There is another type of allergic reaction and that is the ID reaction in which there is a flare-up of related fungi. In this condition, trichophytosis is activated from the injection of penicillin.

Dr. Criepp (Moderator): Well, a moderator is like a quarterback: He calls the signals but every once in a while carries the ball. I shall outline a classification of allergy that is applicable to antibiotics. Immediate: (1) induced or physiologic, such as serum

disease type of reaction and anaphylaxis; (2) the natural, hereditary, or atopic reaction characterized by asthma and urticaria.

The delayed type of reaction is characterized by an eczematous type of contact dermatitis. I do not know whether the panel will entirely agree with this classification. So, we divide all the allergic reactions as Dr. Arbesman pointed out into the immediate and delayed type.

Under the immediate type there are those allergic reactions that are anaphylactic, and the natural type of immediate reactions manifested by asthma. Then, of course, there is the delayed reaction of the non-bacterial type, which Dr. Arbesman referred to as contact dermatitis. Perhaps under this group we can put the hematoimmunologic disturbances that follow the use of antibiotics and perhaps also the collagen diseases that sometimes result from the use of penicillin.

So this is the pattern that we will follow in today's discussion. We will take up the immediate reactions first and then we will consider the delayed response. Now, under the immediate reactions, we are first concerned with the serum disease type. Dr. Sheldon, what is meant by serum disease—let us say to penicillin?

Dr. Sheldon: I think the carry-over from the old days following the administration of horse serum and other types of foreign protein still applies today. That is clinically, as Dr. Arbesman has mentioned, serum disease consists of a trend of symptoms coming on usually 7 to 10 days after the administration of penicillin and characterized by general malaise and fever of varying degrees associated with myalgia and joint symptoms. The presenting symptoms in one patient may be greater in one respect than another and is always characterized by urticaria and/or angioneurotic edema.

Dr. Criepp (Moderator): Is there an immunologic mechanism responsible for this condition?

Dr. Sheldon: Without a question of doubt, it is an immunological response.

Dr. Criepp (Moderator): What do you think actually happens under these circumstances?

Dr. Sheldon: In this instance the individual is building antibodies as a result of antigenic stimulation, in this instance, penicillin. When the crucial point of build-up of antibodies occurs, these react with the antigen (penicillin) so that there appears the clinical picture of serum sickness.

Dr. Criepp (Moderator): This reaction invariably occurs, does it not, Dr. Sheldon, after the administration for the first time of the antigen or penicillin?

Dr. Sheldon: Usually yes. One may have serum sickness appear occasionally after penicillin is given the second or subsequent time. However, it is the usual experience that under these circumstances it will become accelerated. Whereas the first time it may have appeared between the seventh and the tenth day, the second time that it is administered in this given patient there may or may not result serum sickness again. In this instance, it is more liable to occur on the third to the fifth day and then administration on a third occasion after recovery from the first and second serum sickness, the time interval between the development of the symptoms and administration of the medication again becomes shorter and may be a matter of hours or a matter of a few days. It is now referred to as accelerated serum reaction. Lastly, the reaction may be anaphylactic.

Dr. Criepp (Moderator): We will come to that later. So, would you be concerned about the outlook for the patient who has serum disease following penicillin?

Dr. Sheldon: The immediate prognosis is good but the subsequent administration of penicillin to this patient may very well result in a more severe and serious reaction.

Dr. Criepp (Moderator): What are the clinical manifestations in serum disease?

Dr. Sheldon: Usually it is a self-limited disease and the patient gets well in a matter of 7 to 14 days. However, occasionally the individual may develop bronchial plexus paralysis, may develop laryngeal edema and death may ensue, but this is very rare indeed. Occasionally some patients may develop more serious collagen disease.

Dr. Criepp (Moderator): Thank you. In summary of what has been stated about serum disease, it occurs, as Dr. Sheldon pointed out, after the administration for the first time of penicillin. In this condition we are not particularly concerned with the outcome because the condition is usually easily managed with epinephrine, antihistamines, and steroid compounds.

Dr. Weinstein: Dr. Criepp, I would like to ask about the patient who has serum disease that lasts not for one or two weeks but for 3, 4, 6, or 12 months. We sometimes see that too. I would like to have Dr. Sheldon tell us why this should continue to occur.

Dr. Sheldon: We do see this often. I do not think that this is a question that can be completely answered. In some instances, undoubtedly, there is a sufficient amount of circulating antigen to perpetuate the reproduction of additional skin sensitizing reagins and the critical level is repeated time after time over many weeks and months. On the other hand, the individual may also have accidental exposure to minute quantities of penicillin and this may potentiate the urticarial and/or angioneurotic edema response over many, many weeks of time.

Dr. Criepp (Moderator): At any rate, one of the reactions to penicillin is the so-called serum disease type. Now, are there patients who after receiving an antibiotic, particularly penicillin and especially by injection, develop an immediate severe reaction that may be fatal? Dr. Arbesman, how would you classify this kind of immediate reaction? Would you call it an anaphylactic reaction, an anaphylactoid reaction, or an atopic reaction, and why?

Dr. Arbesman: I think this is a play on words really. It is an immediate allergic reaction in the broad sense of the word. If one uses the definition of anaphylaxis as having a primary sensitizing dose and then a secondary challenging dose, then one would have to say that there is no such thing as anaphylaxis in a patient who has his first injection of penicillin. However, somebody else might say "Well, the patient was sensitized by taking small amounts of penicillin found in milk and this acted as a sensitizing dose and then when a challenging dose was given the patient would have the typical anaphylactic reaction." So, as long as we do not definitely know I would prefer to call it anaphylactoid and use the term "anaphylaxis" for guinea pigs and rabbits.

In regard to atopic disease the antibodies must have been originally present. This is usually a familial or hereditary tendency and we know that there is a greater incidence of reaction in patients who are "allergic," that is who are atopic, who have hay fever

and asthma. So whether you call it anaphylactic or anaphylactoid or atopic, it is still an immediate reaction that is fraught with many dangers and often fatalities.

Dr. Criepp (Moderator): What do you conceive to be the mechanism of the production of this serious immediate atopic reaction to penicillin?

Dr. Arbesman: I would assume from this that there are antibodies present against the penicillin before the injection of the penicillin was given. There results antigen antibody union that takes place immediately with release of various chemical mediators, probably histamine, acetylcholine and other chemicals that cause vasodilatation, bronchial spasm, and collapse.

Dr. Criepp (Moderator): Would you venture a guess as to what kind of antibody mediates this atopic reaction?

Dr. Arbesman: We would assume that this might be what is called the skin sensitizing or atopic antibody, the so-called "reagin." However, we are not sure of this.

Dr. Criepp (Moderator): Even in those patients who do not present atopic clinical manifestations, such as asthma, you feel that the skin sensitizing antibodies probably mediate the reaction?

Dr. Arbesman: Probably.

Dr. Criepp (Moderator): What role do you think the other antibodies, such as precipitins and anaphylactic antibodies, sometimes found in these conditions play in mediating these manifestations?

Dr. Arbesman: Unfortunately, there has been little or no work that show antibodies against penicillin in the test tube. However, recently in *Science*, Dr. Allen Leigh reported on a hemagglutination test with penicillinized cells. He found that 25 per cent of the patients that he tested, however, did not have allergic reactions to penicillin, but they still showed a positive hemagglutination test. Recently, I had the privilege of visiting Dr. John Vaughn at the University of Rochester. He used some modifications of Dr. Leigh's hemagglutination technique and found the following. He took the sera from 300 children who were receiving prophylactic doses of penicillin for rheumatic fever. Of these 300 sera, he had only three positive hemagglutination reactions. However, of 18 patients who had generalized or local allergic reactions to penicillin, 14 had positive hemagglutination reactions. This is indeed the first information that I know of that indicates that one can measure this type of antibody in the test tube. Our own experience has been more limited. In 7 patients who had penicillin reactions, we found 4 who had very definite titers against penicillin by this technique. So in time this may promise to be worth something. Now, whether these antibodies were present before they had their reaction or after, of course, is hard to tell. If we were to assume that the anaphylactic reaction was due to the fact that antibodies were already present, then we should be able to get a positive reaction before and not after the allergic reaction takes place. This happens also with other types of antibody that can be measured.

Dr. Criepp (Moderator): Dr. Arbesman, sometimes following the injection of penicillin

there is a massive swelling at the point of injection. Would you suspect that that is similar to the Arthus phenomenon?

Dr. Arbesman: Yes, I could assume that this might be an Arthus type of phenomenon. As a matter of fact, we have all seen this. These patients not only have a marked local reaction but the area breaks down to an ulcer with excoriated margins that are sterile.

Dr. Criepp (Moderator): Would you be brave enough to give a patient who reacted in this manner a subsequent dose of penicillin by injection?

Dr. Arbesman: No, I would not.

Dr. Criepp (Moderator): I certainly agree. We need not go into details on the question of treating these reactions, although that is of great interest. Dr. Siegel, how successful are we in treating these immediate allergic—severe allergic—reactions to penicillin?

Dr. Siegel: The incidence of survival after treatment for the severe immediate reactions, the so-called “anaphylactic reactions,” is quite high. From the reported cases, the incidence is about 10 per cent fatality.

Before we leave the subject of serum disease type of reactions, I do not think we ought to leave the audience with the impression that this is a progressively increasing reaction following subsequent courses of antibiotics. This, of course, is the rule with horse serum. Patients on initial exposure will have a single episode of serum sickness following their first injection. However, following subsequent injections, the interval between the injection and the onset of the serum disease will shorten and finally a condition may actually develop that looks like an anaphylactic reaction. This is one of the differences in the mechanism of penicillin serum sickness-like state and in horse serum disease, in that it does not occur with regularity even in a patient who has received penicillin and who has already had a serum sickness-like reaction therefrom. It is very bizarre but with penicillin it seems to be a transient type of sensitivity that can come and go. Thus, a patient will develop a serum sickness-like state and the penicillin will be continued and the serum sickness will subside.

Dr. Arbesman: I might also augment that statement by the work of Dr. Vaughn, in which he measured the antibodies by the technique I just mentioned and found that after a short period of time the titer of these antibodies gradually lowers and disappears, often within weeks. So they can lose their sensitivity to penicillin.

Dr. Criepp (Moderator): Dr. Sheldon, what do you think about subsequent allergic reactions following initial penicillin serum disease?

Dr. Sheldon: I would agree but I also think that we should not leave the subject without again stating that once a serum sickness reaction has occurred the likelihood of having an anaphylactic reaction or an accelerated type of serum sickness is definitely greater than in the cross section of the population receiving penicillin. So, to me, the fact that they have had a previous type of either local or systemic response is a good warning relative to subsequent administration of this agent.

Dr. Criepp (Moderator): As you see, ladies and gentlemen, we have a little disagreement in our panel. Mr. Knudsen used to say that if two members of his board of

directors always agreed, it was time to fire one of them. However, the fact remains that the serum disease-type of reaction occurs following an incubation period. It is usually manifested by symptoms that were referred to and the management of this condition is usually not difficult. The outlook, i.e., the prognosis, is usually good. In contrast to this, the immediate allergic type of reaction, which manifests itself with atopic symptoms, such as asthma or with circulatory collapse and shock, is serious and therefore to be feared. I am not so optimistic as some of the speakers were with regard to fatalities and perhaps the reason for that is that I have had some bitter experiences in this connection. Such events are dramatic and not easily forgotten. For instance, I can recall the case of a 21 year old nurse who had had a previous serum disease reaction to penicillin. However, she gave herself an injection in the thigh for a cold. In less than five minutes she went into shock and died. This was an anaphylactic reaction.

Dr. Arbesman, is it possible to predict the occurrence of allergy to an antibiotic, such as penicillin by skin testing?

Dr. Arbesman: I think we shall find some disagreement in the panel on this point also. I feel that a skin test is not indicative of sensitivity to penicillin. A positive test may mean something and on the other hand it may not mean a thing. A negative test does not give the reliable results because many patients who have negative skin tests still develop the anaphylactic type of reaction. Now, one thing more. A skin test would mean nothing as far as a serum sickness-like reaction is concerned because you do not have your antibodies beforehand. These antibodies develop 7 to 10 days later. So a skin test would be no indication of serum sickness reaction and I doubt its value in the anaphylactic type.

Dr. Criepp (Moderator): Let us say that you have 10 patients who had a severe, immediate type of penicillin reaction and had had the foresight and the courage to have skin tested them prior to the administration of penicillin. Would you venture a guess as to how many of these 10 patients would have given a positive direct skin test to penicillin?

Dr. Arbesman: I would venture a guess but as far as I know there are very few reports in the literature about this. I would guess that they would all have had skin test reactions beforehand, and some of the skin reactions may have been severe. However, the reports in the literature show positive skin reactions and positive passive transfer are obtained after patients have had this almost fatal type reaction. Dr. Siegel mentioned this noon that he knew of a report in which people were tested before a reaction occurred. Could you tell us about that, Dr. Siegel?

Dr. Siegel: There have been several such instances. The number of positive immediate reactions are always quite small. Among those who were positive, most patients had already had a severe reaction to penicillin. In one case that I know of, the patient had a positive skin reaction to penicillin and a week later through an error was given an injection by another physician and the patient promptly died.

Dr. Criepp (Moderator): We will come back to this in a few minutes. Dr. Sheldon, is sensitization to penicillin in utero possible?

Dr. Sheldon: Yes, it is.

Dr. Criepp (Moderator): Do the antibodies to penicillin pass the placental barrier?

Dr. Sheldon: One must assume they do but so far as I know there has not been any data to prove this.

Dr. Criepp (Moderator): Do you think that you could decide on the basis of a skin test to penicillin whether the patient should or should not get an injection of penicillin?

Dr. Sheldon: My answer to that would be an emphatic no.

Dr. Criepp (Moderator): Then what would you rely on as a safeguard if not on the skin test?

Dr. Sheldon: The procedure that we have available to all of us is the clinical case history and here again this is fraught with certain possible errors. Nevertheless, to me an individual who has had a previous type of reaction, regardless of how mild it might have been, should receive penicillin with caution, if at all. I personally would not give such a patient penicillin.

Dr. Criepp (Moderator): Would you also be hesitant to do a skin test on him?

Dr. Sheldon: I certainly would. Deaths have occurred from skin testing.

Dr. Criepp (Moderator): Suppose you are faced with a patient who needs penicillin badly. He gave a history of having had a reaction to penicillin before. What would you do in this case, Dr. Arbesman?

Dr. Arbesman: There are very few such instances but I can conceive of a patient with subacute bacterial endocarditis who was violently ill and the only antibiotic to which the organism was sensitive was penicillin. In this instance, penicillin must be given and a certain calculated risk must be taken. With this type of patient, a skin test must first be taken. Although I say it is of no value medically, it has medico-legal importance. Then gradually increasing doses every 15 minutes must be given until the proper therapeutic level is obtained. The patient is protected beforehand by antihistamines, epinephrine, and possibly hydrocortisone. These agents are also made available for immediate use. In the cases that we have tried this we have been successful each time. This brings up the question did we de-sensitize these patients by this split dosage and gradual increased dosages or did we do something else? Did these patients have an anergic phase due to a high fever and did not react or did they lose their sensitivity as we talked about before?

Dr. Criepp (Moderator): You really do not think you de-sensitized them, do you?

Dr. Arbesman: No, I really do not. I think we were just lucky and got away with it.

Dr. Criepp (Moderator): Dr. Siegel, penicillin is not a protein. How does it become antigenic?

Dr. Siegel: Of course, there have been some conjectures that penicillin acts as a hapten, that is penicillin combines with some of the body proteins and this combination is antigenic. However, as you know penicillin is a very poor antigen in animals. When one combines the penicillin with plasma proteins and uses this combination in sensitizing animals there is no increased effectiveness as an antigen.

Dr. Criepp (Moderator): Dr. Weinstein, I think you took exception to at least some of the remarks that Dr. Arbesman made with reference to the treatment of patients who need penicillin. Did you not say that there are other antibiotics that can be used in place of penicillin, even in a case of subacute bacterial endocarditis?

Dr. Weinstein: I agreed with everything that Dr. Arbesman said except that I have never seen any infection or any bacteria that was sensitive to penicillin and to penicillin alone and to no other antibiotic. I think that there are very few, if any, cases in which penicillin is the only drug that can be used in treating a given disease.

Dr. Criepp (Moderator): What would you give this patient with subacute bacterial endocarditis?

Dr. Weinstein: I would give one of the other antibiotics that I am certain—this patient, of course, is sensitive to penicillin—this organism would be sensitive to, after determining its sensitivity, of course.

Dr. Arbesman: I take some objection to that because I think there are certain instances in which only penicillin is the drug of choice and the one you have got to use. In this type of patient, these other antibiotics have been used and the patient is still sick and still has colonies in his blood cultures. You see such a situation all the time and penicillin is the only drug that is effective. We have seen this many times. Give the patient large doses of chloramphenicol, for instance, and they still are sick; but give them penicillin, and they get better.

Dr. Weinstein: I have to agree with Dr. Arbesman. If he postulates that there exists such cases in which penicillin alone is the only drug that can be used, there just is no other choice.

Dr. Criepp (Moderator): But you are still skeptical.

Dr. Weinstein: I still do not think that is true.

Dr. Criepp (Moderator): I think someone wisely said "skepticism is the chastity of the intellectual, it should not be surrendered lightly."

Dr. Sheldon, if you transfuse blood from a penicillin-sensitive patient to a normal recipient, would the recipient become allergic to penicillin?

Dr. Sheldon: Temporarily.

Dr. Criepp (Moderator): So if he got an injection or was exposed to penicillin he would develop a reaction.

Dr. Sheldon: That is right.

Dr. Criepp (Moderator): Well, gentlemen, can these patients be given penicillin by mouth, where there is a need for it, and thus take less of a chance than by giving it by injection? Dr. Arbesman?

Dr. Arbesman: I think that you can get away with penicillin by mouth a lot more

readily than you can by injection but there are still fatalities reported by giving it orally, although the incidence of it is much less.

In some of these cases of subacute bacterial endocarditis, you would have to give the patients a barrel of pills to make them better.

Dr. Weinstein: Dr. Arbesman, if I remember correctly, there were cases of anaphylactoid shock due to oral penicillin, for that matter due to penicillin ointments, but I do not know of any case of fatality due to oral penicillin.

Dr. Arbesman: Yes, they have been reported.

Dr. Criepp (Moderator): There have been deaths reported to oral penicillin but they are certainly not anywhere near as common as those from injected penicillin. Of course, the truth of the matter is that we have to rely on the history and yet we know that people are garrulous and frequently the history is not too reliable, especially when you deal with—this term is no longer permissible in this day of psychosomatics—the neurotic patient.

Incidentally, when you use penicillin for skin testing, how much penicillin do you use, Dr. Sheldon?

Dr. Sheldon: I, frankly, use the intracutaneous technique. I think it is the most reliable technique to determine whether a patient has systemic sensitivity. Now, what is a safe starting dose in doing these tests? I rely on my history. If the patient has ever had a shock reaction or an immediate reaction to penicillin, I will not test that patient at all. I will, instead, do what is known as a passive transfer test. I shall explain that in a moment. If I do not get the history of an immediate reaction, I will start with approximately 0.5 to 1 unit of penicillin intracutaneously and move up 100-fold at a time until I have reached approximately 50,000 units/ml. If the patient does not respond to that, then I consider him negative.

Dr. Criepp (Moderator): An interesting thing is that, unless I am wrong, skin testing with drugs is notoriously unreliable. Am I right about that? Therefore, why should skin testing with penicillin be more reliable than skin testing with aspirin, for instance?

Dr. Sheldon: No one has ever been able to demonstrate the skin sensitizing antibody for most drugs. In the case of penicillin we do know that some individuals do have a positive skin reaction. Other drugs, quinine, for example, will give a good positive skin reaction, i.e., an individual who has systemic disease of allergic nature from administration of quinine. I am of the same school of thought as Dr. Arbesman. I have no fight with Dr. Siegel, however, for I just do not test whenever there is a question raised.

Dr. Criepp (Moderator): Has any member of the panel had any reaction from the administration of poliomyelitis vaccine because of penicillin sensitivity?

Dr. Siegel: Yes.

Dr. Criepp (Moderator): Would you briefly state what the reaction was?

Dr. Siegel: An immediate constitutional reaction following 1 ml. injection of poliomyelitis vaccine, which was supposed to contain less than .003 unit/ml. of penicillin.

Dr. Sheldon: Can we be positive that this was due to penicillin, Dr. Siegel? Are there any other ingredients, such as horse serum, that might have been present?

Dr. Siegel: In view of the fact that I produced the same reaction in this patient with a skin test containing 0.5 unit of penicillin, I think that the penicillin was responsible in the poliomyelitis vaccine.

Dr. Criepp (Moderator): So much for that. It is obvious that patients who are sufficiently sensitive to a product will give a reaction, especially if they are exposed to that product by injection. Is there any poliomyelitis vaccine made without penicillin, Dr. Weinstein?

Dr. Weinstein: Yes, most of the poliomyelitis vaccine today is made without penicillin. Some of it is made with streptomycin as the bacteriostatic agent.

Dr. Arbesman: There has been a report in a recent issue of the *Journal of the American Medical Association*, in which the Eli Lilly Company stated that 80 per cent of the batches of their poliomyelitis vaccine contained less than .001 mg./ml. of penicillin and that 100 per cent contained less than 1 mg./ml. The incidence of reactions is very interesting in this report in that, of 184 million doses of poliomyelitis vaccine that were given, there were only 56 allergic reactions, of which 4 were of the anaphylactic type.

Dr. Criepp (Moderator): Do any of the members of the panel use penicillinase in the treatment of very severe immediate reactions? Dr. Weinstein, have you had any experience with it?

Dr. Weinstein: Not in the treatment of immediate reactions.

Dr. Criepp (Moderator): I am talking about immediate reactions. What is the mechanism of action of penicillinase?

Dr. Weinstein: Penicillinase is an enzyme, a protein molecule derived for commercial purposes at present from *Bacillus cereus*. It is also produced by other organisms, such as *B. subtilis* and certain staphylococci, especially those penicillin resistant. Penicillinase acts as a catalyst in the hydrolysis of penicillin, which is broken at the beta-lactam ring to form penicilloic acid which I thought, up till about three hours ago, was nonallergenic. However, Dr. Sheldon knows otherwise and I would like to hear from him.

Dr. Sheldon: I should like to quote in regard to the relationship of the presence of penicilloic acid in patients with generalized exfoliating erythroderm. Dr. Herman Eisen has reported verbally to me on this. I do not know that this is in the literature, namely, that in this skin condition occurring after the administration of penicillin this chemical agent is present. If this is true, then it would be counterindicated to give penicillinase in individuals who have exfoliating erythroderm.

Dr. Criepp (Moderator): Has anybody had any experience with allergic reactions to penicillinase?

Dr. Arbesman: I have not had any but there are several reported cases of allergic reactions to penicillinase. People can get anaphylactic type reactions from this too. This has

been produced in animals also. It is possible to sensitize animals to penicillinase and shock them. So you may be treating the penicillin reaction and get a worse reaction than you had before.

Dr. Criepp (Moderator): Would any members of the panel suspect that it might be possible to administer penicillinase to an allergic patient who needs penicillin, Dr. Weinstein to the contrary notwithstanding, and thus give it to him prophylactically?

Dr. Weinstein: It would not work.

Dr. Criepp (Moderator): That is exactly what I hoped you would bring out. How soon after the administration of penicillinase does penicillin disappear from the circulation, Dr. Weinstein?

Dr. Weinstein: Penicillinase is standardized in the so-called Levy units whereby one unit of penicillinase inactivates one unit of penicillin per minute or 60 units/hour at 25 C. at pH 7. This is in vitro and is a little faster in vivo.

Dr. Arbesman: I have an interesting comment about that. I had a neurosurgeon patient who was given penicillin for streptococcal sore throat and he developed a delayed type of reaction from the penicillin and was given penicillinase and his sore throat and fever came back but his allergic reaction continued.

Dr. Criepp (Moderator): This has nothing to do with allergy but I am very much tempted to ask Dr. Weinstein this question. Do you think that as a result of the promiscuous use of penicillin other organisms besides the *Staphylococcus* may become penicillin resistant?

Dr. Weinstein: Yes, there are other organisms that not only become penicillin resistant but that overgrow the penicillin-sensitive organisms that normally keep them in check. One of the most frequent occurrences are the *Candida* infections following the use of penicillin, as well as colon bacilli. Many bacilli that are not sensitive to penicillin will take over when the penicillin-sensitive strains are knocked out.

Dr. Criepp (Moderator): Penicillin has gotten the brunt of most of this discussion. I would like to pass on to the question of immediate reactions to streptomycin. Dr. Weinstein, can you comment on that?

Dr. Weinstein: The immediate reactions to streptomycin are usually manifested in the form of drug fever and skin eruptions. Of course, the later results that may come on after several weeks exposure are the vestibular portion of the acoustic nerve damage, vertigo.

Dr. Criepp (Moderator): You think that is an allergic reaction?

Dr. Weinstein: No. Allergic reactions to streptomycin are mostly the skin manifestations.

Dr. Criepp (Moderator): Has anybody on the panel had any allergic reactions to streptomycin? What makes one agent a potent allergen and another one not so potent? Dr. Arbesman?

Dr. Arbesman: I do not know.

Dr. Criepp (Moderator): Can one anticipate allergenicity of a drug?

Dr. Arbesman: Theoretically you can tell by the chemical structure of the drugs, and very often drugs with benzene rings are potential sensitizers. However, there are many difficulties.

Dr. Criepp (Moderator): Dr. Siegel, do you think that any agent is likely to become allergenic with increased use?

Dr. Siegel: Yes, I do. For instance, the problem is now raised about some of our new synthetic penicillins, which might not react in our known penicillin-sensitive cases. I think it is quite possible that with continued use we will begin to see the same type of pattern now seen.

Dr. Criepp (Moderator): Do patients with agammaglobulinemia develop penicillin allergy, Dr. Sheldon?

Dr. Sheldon: Yes, they do.

Dr. Criepp (Moderator): Have you had any experience with that?

Dr. Sheldon: Not personal experience but according to literature and some of my pediatric friends particularly, this has occurred.

Dr. Criepp (Moderator): That is any kind of an allergic reaction?

Dr. Sheldon: Well, you speak of agammaglobulinemia, but in most instances it is not agammaglobulinemia: it is a hypogammaglobulinemia. Furthermore, it is entirely possible that antibodies are not necessarily carried in the gamma globulin fraction exclusively.

Dr. Criepp (Moderator): Dr. Siegel made reference to synthetic penicillin. Dr. Weinstein, what would be your guess with regard to the allergenicity of a penicillin product that is synthetic?

Dr. Weinstein: I do not think that the fact that it is synthetic, or made by fermentation process, affects its allergenicity. The so-called synthetic penicillin, α -phenoxethyl penicillin, is very closely related chemically to penicillin V, which is α -phenoxymethyl penicillin. Although to date there have been few if any reports of allergic reactions to this drug, I would assume that in time we might see some reactions to it.

Dr. Criepp (Moderator): Would you suspect that there is enough of a change in a chemical formula of this product to make it possible to administer it to a patient who is sensitive to other penicillin products?

Dr. Weinstein: Yes, it has been done. It has been administered to patients who were allergic to other penicillins without any untoward effect.

Dr. Arbesman: They may have lost their sensitivity.

Dr. Weinstein: That is true.

Dr. Criepp (Moderator): So much then for the immediate allergic reactions to penicillin and to other antibiotics. They are the serum disease type, which is relatively innocuous, and the immediate type of allergic reactions, which may be anaphylactoid; or they may be atopic and much more serious. In these cases the outlook is fairly bad and, unless one starts treatment before the patient's condition reaches the point of no return, treatment avails very little in my opinion.

Clinicians are very sensitive about discussing treatment before an audience composed of researchers, but perhaps we should spend a few minutes on treatment, if the research men here will be indulgent. Dr. Arbesman has already referred to the treatment of the serum disease type of reaction, which is symptomatic. Dr. Siegel, how would you treat such a patient who develops immediate severe serious reaction to penicillin?

Dr. Siegel: First choice would be epinephrine. This should be given in doses of either 0.5 or 1 ml., either subcutaneously, intravenously, or perhaps even intracardially, depending on the status of your patient. These doses should be repeated, depending upon the condition of the patient. If shock is profound, it may be necessary to use levarterenol bitartrate intravenously, or blood transfusions of plasma if necessary or available. If the injection has been given into the arm, it might be a good idea to place a tourniquet above the site of injection to slow down the absorption of the penicillin, though for the most part the damage has already been done. Oxygen is given in those cases that are cyanotic or having dyspnea or asthmatic breathing. Intravenous or subcutaneous antihistamines have a place only when you want to relieve some of the pruritus but should not be relied upon for the patient who is in shock. The same holds true for steroids, which should not be relied upon for the patient who has an anaphylactic shock reaction. This takes too long to be effective. It may be of value in preventing some of the recurrences that occasionally occur but not for the immediate status.

Dr. Criepp (Moderator): Now let us pass on to contact dermatitis, which is a delayed form of allergic reaction to antibiotics. Dr. Siegel, is there any danger in development of contact dermatitis in individuals who handle antibiotics?

Dr. Siegel: Yes, I think the people in the audience could answer that better. I understand that in the pharmaceutical industry, in areas where penicillin, for instance, is handled a good deal, this is quite a problem and the workers have to be shifted around frequently. I am told that among people who handle antibiotics a very high percentage of them will develop positive patch tests even if they have not yet developed the contact dermatitis.

Dr. Criepp (Moderator): Then diagnosis is made by means of a patch test?

Dr. Siegel: Yes, sir.

Dr. Criepp (Moderator): What is the immunologic mechanism that mediates contact dermatitis, Dr. Siegel?

Dr. Siegel: That is not completely known but the contact dermatitis is an epidermal sensitization rather than a systemic sensitization. A local sensitivity develops at the area of contact, which spreads over the entire epidermal surface. In some cases, a trans-

fer of this sensitivity by means of leukocytes has occurred from patients who have positive contact reactions to patients who are normal.

Dr. Criepp (Moderator): Dr. Weinstein, besides penicillin and streptomycin, are there any other antibiotics that can lead to contact dermatitis?

Dr. Weinstein: Yes, there are other antibiotics that are not used in human medicine, for example cycloheximide has been known to cause dermatitis. As a matter of fact, we heard a paper on that very subject earlier this afternoon. It causes the dermatitis in the handlers who spray trees and plants to get rid of plant rust. Streptomycin is also used for that purpose and dermatitis ensues. Of course, penicillin might cause it but not frequently.

Dr. Criepp (Moderator): Dr. Siegel, you suspect then that a cellular antibody probably mediates contact dermatitis?

Dr. Siegel: That is the suspicion today.

Dr. Criepp (Moderator): Dr. Sheldon, what is collagen disease?

Dr. Sheldon: That is a group of clinical entities in which there is uniform pathological findings of fragmentation of collagen fibers with inflammatory response particularly in and about the small vascular bed.

Dr. Criepp (Moderator): What diseases may be grouped under this heading?

Dr. Sheldon: There are a number of them. The outstanding ones are disseminated lupus erythematosus, periarteritis nodosa, perhaps rheumatoid arthritis, certain types of nephritis, particularly the hemorrhagic type of nephritis, and perhaps dermatomyositis.

Dr. Criepp (Moderator): Do you think that periarteritis nodosa might be an allergic disease in the sense of delayed allergy?

Dr. Sheldon: Yes.

Dr. Criepp (Moderator): What are the implicated antigens?

Dr. Sheldon: There are a number following any serum sickness disease. They have been noted frequently after using some of the chemotherapeutic agents, particularly the sulfonamides, and after administration of iodides.

Dr. Criepp (Moderator): What about antibiotics?

Dr. Sheldon: Yes, after penicillin and particularly after penicillin has resulted in a serum sickness reaction followed by the collagen changes.

Dr. Criepp (Moderator): What is there, Dr. Arbesman, that confirms or justifies our suspicion that periarteritis nodosa may well be a disease of hypersensitiveness?

Dr. Arbesman: Going back many years, Dr. Rich of Johns Hopkins showed that when he injected large doses of horse serum in rabbits they would develop vascular lesions that were very similar to that of periarteritis nodosa. Dr. Germuth in more recent years has been able to do the same thing and these lesions, as Dr. Criepp has shown, can be inhibited by giving steroids prior to the injection of the horse serum. However, once the serum has been given, the corticosteroids will not prevent these lesions.

Dr. Criepp (Moderator): Also, periarteritis nodosa is frequently associated with intractable bronchial asthma with eosinophilia. It responds, as do all allergic diseases, to the administration of steroids. We have shown that the patient has hypergammaglobulemia. Gamma globulin has been demonstrated in the pathological lesions both experimentally and clinically, so that hypersensitiveness offers the only clue thus far to the possible etiology of this group of diseases. I have seen a case that developed periarteritis nodosa following the use of penicillin for the treatment of syphilis; I also have a case, and I understand that Dr. Arbesman has one also, of dermatomyositis, which also developed following the use of penicillin.

Dr. Arbesman: I might say, however, that the corticosteroids have been reported to produce periarteritis.

Dr. Criepp (Moderator): That is right. Now, what is exfoliating dermatitis, Dr. Sheldon?

Dr. Sheldon: Exfoliating dermatitis is spoken of commonly as an exfoliating erythroderm and it is a generalized erythematous eruption of the skin in which the patient desquamates.

Dr. Criepp (Moderator): Dr. Sheldon, with reference to immuno-hematologic diseases, have you had any experience with penicillin or other antibiotics producing hematologic disorders of this type and what were they?

Dr. Sheldon: Yes, there are a number of entities that I presume you and the rest of the panel might argue as to whether it is a real allergic reaction or not, but agranulocytosis, is one condition that may follow the administration of certain antibiotics.

Dr. Criepp (Moderator): Would you venture a guess as to how an antibiotic, say penicillin, produces thrombocytopenic purpura? How does it act as an antigen and what is its effect on the platelet?

Dr. Sheldon: Certainly it has a suppressing effect and probably the action may well be upon the bone marrow or other portions of the hemopoietic system.

Dr. Criepp (Moderator): Do you believe that that action is similar to apronal or quinine on the platelet? Does it activate the antigenicity of the platelet?

Dr. Sheldon: Probably, although you cannot demonstrate it as you can in the case of quinidine or apronal.

Dr. Criepp (Moderator): Is there presumptive evidence to this effect?

Dr. Sheldon: There is presumptive evidence only.

Dr. Crieep (Moderator): Dr. Arbesman, do you want to comment on that?

Dr. Arbesman: No, I feel that we are merely theorizing and we do not know the answer to that.

Dr. Crieep (Moderator): Would any member of the panel have any opinion as to whether hepatic or renal dysfunction following the use of penicillin, or perhaps another antibiotic, classifies as an allergic disorder?

Dr. Arbesman: I do not believe it is an allergic disorder.

Dr. Crieep (Moderator): You do not believe that the renal changes that are seen may be analogous to those that we see following experimental serum disease nephritis?

Dr. Arbesman: Here again you can theorize, but we have no proof; unfortunately we do not have a method of measuring the antibodies properly.

Dr. Crieep (Moderator): Have you had any instances of drug fever following the administration of antibiotics and is this an allergic manifestation?

Dr. Arbesman: I have had cases of drug fever from the sulfonamides and also from penicillin.

Dr. Crieep (Moderator): Has anybody here seen a drug fever type of reaction to antibiotics?

Dr. Siegel: It has been described with streptomycin.

Dr. Crieep (Moderator): What about cross reactivity, Dr. Weinstein? You have already touched upon this. I would like to know whether penicillin cross reacts antigenically with anything else?

Dr. Weinstein: To my knowledge, it does not react cross antigenically with anything else except with other penicillins. Penicillin V and G, for example, are cross antigenic. Synthetic penicillin, as brought out in one of the earlier papers, is less so. However, I do not know of any other drug that demonstrates cross antigenicity to penicillin.

Dr. Crieep (Moderator): What about the broad-spectrum antibiotics and their cross antigenicity?

Dr. Weinstein: The tetracyclines definitely show cross antigenicity: if one is sensitive to one of the tetracyclines, he is usually sensitive to all of them. This does not hold for chloramphenicol, which is also one of the so-called "broad-spectrum antibiotics."

Dr. Crieep (Moderator): Do you believe that sensitivity to *Penicillium* means sensitivity to penicillin?

Dr. Weinstein: No, sir.

Dr. Sheldon: Dr. Crieep, I think that is an important point because so commonly patients

wonder about the fact that they have hay fever and/or asthma due to allergy to the spores of *Penicillium* blowing in the air. They invariably ask the question "Does this mean that I am sensitive to penicillin?" Since this has been raised so often, I think it is a point worthwhile emphasizing.

Dr. Criepp (Moderator): Dr. Arbesman, do you believe there is any relation between penicillin allergy and dermatophytosis?

Dr. Arbesman: Well, there are many cases reported where a person who has a latent ringworm and is given an injection of penicillin gets a penicillin reaction.

Dr. Criepp (Moderator): Do you think they are cross antigenic?

Dr. Arbesman: Yes.

Dr. Criepp (Moderator): Dr. Weinstein, do you agree to that?

Dr. Weinstein: Yes, I do.

Dr. Siegel: I would like to disagree. Among patients who have had epidermophytosis the trichophytin test is positive in more than 50 per cent. A very small percentage actually develop penicillin reactions. If there was much cross antigenicity, we should see more reactions.

Dr. Criepp (Moderator): The next topic has to do with the availability of penicillin in milk. Dr. Weinstein, are there any other antibiotics used for the treatment of mastitis in cows in addition to penicillin?

Dr. Weinstein: Yes. Most of the antibiotics are used in the treatment of mastitis. In fact all the antibiotics that are used in human medicine are also used in the treatment of mastitis.

Dr. Criepp (Moderator): Does milk contain penicillin because farmers add penicillin directly to milk to prevent spoilage?

Dr. Weinstein: No, the penicillin in the milk is there because the cows are milked too soon after the penicillin is injected into the udder. Actually they should wait at least three to four days, and discard the milk during that time. It is an economic problem with the farmers and the penicillin that was in the udder of the cow is transferred to the milk.

Dr. Criepp (Moderator): Dr. Siegel, do you believe that the amount of penicillin that has been demonstrated to be present in samples of milk as per report by Dr. Welch and his associates is sufficient to be a hazard?

Dr. Siegel: Yes, I do.

Dr. Criepp (Moderator): You believe it is a hazard for people who are penicillin sensitive or do you think it is also a hazard from the point of view of sensitizing individuals to penicillin?

Dr. Siegel: I agree to both.

Dr. Criepp (Moderator): To both?

Dr. Siegel: Yes, sir.

Dr. Criepp (Moderator): In other words, do you think that the measures that have been taken by the Food and Drug Administration have been effective thus far?

Dr. Siegel: They certainly appear to be effective. The last survey done showed a marked decrease in the incidence of contaminated specimens and the range of contamination was a little higher but that was because they tested raw milk specimens rather than pasteurized.

Data obtained from Dr. Welch and his group show examples of the heavier penicillin contaminations of milk samples in the 1956 survey. Using their figures, we, of course, were interested to find out whether such amounts could cause reactions in sensitive individuals and by the same token we felt that it was quite hazardous to feed them purposely to sensitive individuals, since we could produce very severe reactions. So we proceeded to do what we call passive transfer tests. In other words we took serum from a patient who was extremely sensitive to penicillin and this patient had reagins against penicillin in his blood stream. This serum was injected into the skin of normal patients who on a fasting stomach were fed penicillin. With 100 units of penicillin G by mouth, a patient developed a 2 plus reaction at the sensitized site. In other words, it was absorbed and was carried to the sensitized site and caused the reaction there. Then we gradually decreased the doses to 50, 40, 37.5, and 25 units. Forty units by mouth was the smallest dose that could cause a positive reaction. We repeated the study using intravenous doses of penicillin and the minimal dose necessary to cause a flare-up of a passively sensitized site was one third less than the oral. In other words, it was about a three to one ratio. Of course, a patient, such as the one who provided us with this serum, could very easily be disturbed by the amounts of penicillin found in those specimens.

We must conjecture that patients who are very sensitive to penicillin, such as the anaphylactic types of sensitivity, the immediate reactors, can very easily be disturbed by the amounts of penicillin in contaminated milk specimens.

Dr. Criepp (Moderator): I understand that antibiotics are used for other purposes, spraying of vegetables and for the preservation of fish and poultry and for growth purposes in cattle and herds and so on. Do you have any opinion whether antibiotics used in that way hold any health hazard? Dr. Siegel?

Dr. Siegel: It is my understanding that the tetracyclines are used primarily for that purpose. In considering the incidence of tetracycline sensitivity as against penicillin sensitivity, it becomes a very minute problem.

Dr. Criepp (Moderator): Dr. Weinstein?

Dr. Weinstein: I agree with Dr. Siegel. The only antibiotics permissible as food preservatives are chlortetracycline and oxytetracycline in poultry and chlortetracycline in fish. In poultry the tolerances for the raw bird is seven parts per million in any part of the bird and after it is cooked or prepared there is no antibiotic residue whatsoever. In fish there is very little residue, in fact almost none.

Dr. Criepp (Moderator): What is the cross reactivity between penicillin and erythromycin?

Dr. Weinstein: None to my knowledge.

Dr. Criepp (Moderator): Is photosensitivity a factor?

Dr. Arbesman: There are cases reported of disseminated lupus developing following penicillin reaction and in such cases, as you all know, the patient is very photosensitive.

Dr. Criepp (Moderator): I do not believe that desensitization to any drug is a practical procedure. Dr. Sheldon?

Dr. Sheldon: There is no question that it is effective in a high percentage of individuals. Our experience has been much less than that of Dr. Arbesman. We have used it in a number of people and it is effective in controlling promptly a serum sickness reaction.

Dr. Arbesman: I cannot go along with that. Perhaps 50 per cent of them get better with penicillinase but that is all. Those patients whom you really want to use it on do not respond at all.

Dr. Criepp (Moderator): What about the reaction between penicillin and *Penicillium*?

Dr. Siegel: There is no cross reaction between these two antigens. It has been suggested that patients who are sensitive to penicillin refrain from eating blue cheese. That is a fallacy.

Dr. Criepp (Moderator): (Question from the floor) What about using penicillin O in place of penicillin G?

Dr. Arbesman: In most instances there are comparable reactions, especially in delayed type cases in which you are not sure that they would have had another reaction even if they had been given penicillin G.

Dr. Criepp (Moderator): This ends the discussion. We have tried to cover the nature and mechanisms as well as the incidence of allergic reactions to antibiotics.

I am grateful to the members of the Panel for their generous contribution of time and skill, and we thank you, a wonderful and attentive audience, for your patience.

The Chemotherapy of Acute and Chronic Pediatric and Geriatric Infections

Panel Discussion

Sydney Ross, Moderator

Douglas H. Sprunt, Moderator

Members of the Panel

Sander Goodman
Robert H. High

Mark H. Lepper
Charles Weiss

Dr. Welch: The panel discussion this afternoon is going to be on the chemotherapy of acute and chronic pediatric and geriatric infections. We are very fortunate, I believe, in our moderators. Dr. Sydney Ross, who is Associate Professor of Pediatrics at Georgetown and Chief of Microbiology at Children's Hospital here in Washington, D.C., will moderate that portion of the panel on pediatric infections. Dr. Douglas H. Sprunt, Professor of Pathology at the University of Tennessee's College of Medicine, will moderate the panel on geriatric infections. I think you will find this a most interesting panel discussion.

Dr. Ross (Moderator): I would like to introduce the panelists on our part of the program: Dr. Robert High, from Temple University Medical School, and Dr. Charles Weiss from Detroit.

Dr. Sprunt (Moderator): I would like to introduce the other two panelists: Dr. Sander Goodman, Assistant Professor, University of Cincinnati College of Medicine, and Dr. Mark Lepper, Professor of Preventive Medicine, University of Illinois College of Medicine.

Dr. Ross (Moderator): In this panel this afternoon, we shall attempt, by a process of sort of a morganatic marriage, to cover both ends of the age spectrum. As Dr. Goodman mentioned before the meeting, there are certain things that the geriatric and the pediatric patient possess in common and these facets will be elaborated upon as the panel progresses.

By way of prologue, there are certain points that I would just like to discuss regarding the use of antibiotics in the pediatric age group.

One gets the impression (without any clear-cut statistical proof) that antibiotics are perhaps used more indiscriminately in children than in other patients. The reason is understandable even though one cannot condone the practice. The most common infections seen in pediatrics are the upper respiratory infections. The incidence of respiratory complications such as otitis media, cervical adenitis, bronchitis and pneumonia is inordinately high, especially in infants and young children. The pediatrician or general practitioner, seeing the child at home or in the office with an upper respiratory infection, is not likely to perform any bacteriological laboratory studies to determine the inciting bacterial agent. Nor is a white blood cell count frequently invoked as a diagnostic aid. These studies would be routine with a hospitalized child, but the vast majority of children

(approximately 95 per cent) receiving antibiotics are treated outside the hospital. Thus, the physician, seeing the child on a house or office visit and armed only with a stethoscope, otoscope, and tongue blade, must make a quick decision, based entirely on clinical findings, whether to give an antibiotic or withhold it. A clinical estimate of whether or not a respiratory infection is due to a bacterial agent that will respond to antibiotics or a viral agent that will not, is at best only a rough guess. Thus the temptation is strong to give antibiotics in any such equivocal situation so as "not to take any chances." This practice of giving antibiotics when in doubt is further intensified by the overt or covert pressure on the part of the parents to give the child "a shot of penicillin" or perhaps one of the "wonder drugs." Although I believe strongly that no physician should be stampeded into a course of therapy against his judgement, any physician doing pediatric practice can attest to the oppressive proportions that this latter consideration can at times assume.

These are some of the factors that perhaps account for the excessive use of antibiotics in the pediatric age group. I would perhaps prefer to use the term "excessive" rather than "indiscriminate," since the latter has a somewhat sterile connotation in view of the difficulties inherent in a situation in which only clinical indices are available to help decide whether an antibiotic is being used with or without adequate reason. In brief, one must probably accept the premise that the discriminating use of antibiotics is frequently predicated on adequate laboratory facilities and that the latter are not generally used except in the hospitalized patient. This does not preclude intelligent use of antibiotics in the home or in the office, but admittedly makes it somewhat more difficult when only clinical criteria are available. The rapid defervescence of fever in a self-limited virus respiratory infection is frequently accepted *a priori* by both physician and parent alike as the direct therapeutic result of "a shot of penicillin" given the day before.

I would like to call on our first panelist to discuss the problem of antibiotic administration in the neonatal period. As you well know, there are certain drugs with an alcoholic or phenolic hydroxyl group that require glucuronide conjugation for excretion. It might be well to point out that in the newborn, glucuronic acid approximates only 30 to 50 per cent of the amount observed several weeks postpartum. You have all heard of the so-called "gray syndrome" and the question of how readily you can administer certain antibiotics to the neonate with impunity, in view of impaired glucuronide conjugation, is a point of vital interest to all of us who handle children. I'd like to call on Dr. Charles Weiss to discuss this problem. Dr. Weiss.

Dr. Weiss: Thank you. During the past few years there has been increasing concern regarding the rising frequency of nursery infections. As a result, a more general use of antibiotics for prophylaxis of these infections as well as for specific therapy has occurred. Chloramphenicol has been a most frequent choice because of its general effectiveness against the more common hospital strains of microorganisms. Doses much higher than those recommended for older subjects have been used in many instances. Associated with the higher doses of chloramphenicol several pediatric centers found a higher incidence of toxic reactions, as well as death in some instances in the premature and newborn age groups. As these observations were reported to us, they were promptly investigated. Careful study of case histories and preliminary laboratory data resulted in the following common observations. In most cases, therapy with chloramphenicol had been instituted within the first 48 hours of life. Symptoms first appeared after three to four days of continued treatment with high doses of chloramphenicol, that is, 100 mg./Kg. daily or more. The symptoms appeared in the following order: abdominal distension with or without emesis, progressive pallid cyanosis, vasomotor collapse frequently accompanied by irregular respiration. Death occurred within a few hours of onset of

these symptoms in some cases. This has been referred to in some institutions as the "gray syndrome." The progression of symptoms from onset to exitus was accelerated with higher dose schedules. Preliminary blood serum level studies revealed unusually high concentrations of chloramphenicol after repeated doses. No characteristic pathological changes attributable to the use of chloramphenicol were found in any of the organ systems, including the hematopoietic system. Termination of therapy upon early evidence of associated symptomatology frequently reversed the process with complete recovery.

The most direct approach to this problem seemed to be through determination of chloramphenicol blood levels. Dr. Glazko, of our laboratories, devised a colorimetric procedure permitting the use of capillary blood. This permitted serial specimens to be drawn, even in the smallest of subjects. Cooperative studies were instituted in a number of pediatric centers in which blood specimens were collected and sent to us for analysis. In January of this year, a general letter from the Director of Clinical Investigation, Parke, Davis & Company, was sent to every physician in the United States and Canada, apprising them of this situation. This letter, based upon early laboratory data and pediatric consultation, recommended a maximum dose of 50 mg./Kg./24 hours for full-term newborn infants up to the age of 1 month. One half of this dose was recommended for infants of the premature age group. Subsequently, no reports relative to this condition have been brought to our attention. With no reports of syndrome in infants older than 1 month of age, the usual dose recommendations were maintained beyond this age level. The blood level determination was advised as a guide to therapy. The colorimetric technique and the chloramphenicol standards were made available to physicians and laboratories requesting them.

The program of investigation was then intensified. Blood levels in considerable numbers were obtained with different chloramphenicol preparations and varied dose schedules. Urinary excretion information was also collected in infants while on such test studies. All blood specimens were forwarded to our laboratories where colorimetric determination of total nitro compounds was accomplished. All results were expressed in terms of chloramphenicol equivalents. The colorimetric procedure with whole blood gave levels that were roughly 80 per cent of those obtained with plasma or serum. The differences in levels may then be influenced by the proportion of free drug to the glucuronide form and also by the packed cell volume. It would have been desirable to include microbioassays on all specimens in parallel with the colorimetric assays. However, this was not possible due to the high dilution of the blood filtrate. Numerous spot tests were accomplished wherever possible by the turbidimetric procedure against *Shigella sonnei*. The microbiological assays were about 65 per cent of the colorimetric values in infants, compared to 90 per cent in adults. This indicated that the blood of infants receiving chloramphenicol contained a higher proportion of inactive metabolic products detectable by the colorimetric procedure but not by the microbial assay. Although the microbial assay provides essential information for the effective levels of free chloramphenicol, the colorimetric procedure is more useful in this study in order that a clearer picture of over-all absorption and excretion of this antibiotic might be obtained.

The product forms of chloramphenicol were studied separately and will be considered in the same manner. There are pronounced differences in their absorption and behavior in the body. For obvious reasons, capsule material was not studied.

Chloramphenicol palmitate will first be discussed. This water soluble ester is marketed as a liquid suspension for oral administration. Precise doses were administered orally to infants with a tuberculin syringe. Each dose was followed immediately with glucose-water to insure complete ingestion. In one series of observations, the blood levels were studied in two different age groups. One group consisted of 13 children ranging from 1 to 12 years of age. A second group consisted of 5 newborn infants under 48

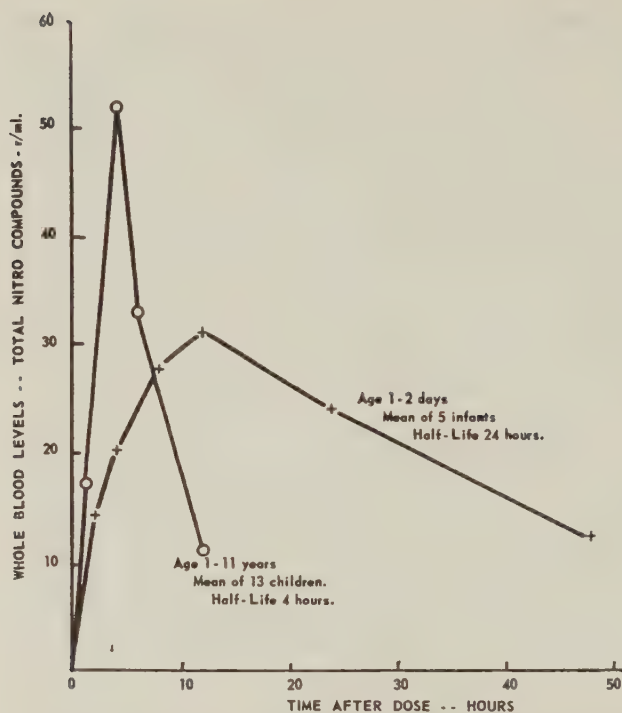


FIG. 1. Results of a single oral dose of chloramphenicol palmitate suspension, 50 mg./Kg.

hours of age. Each subject received a single oral dose of chloramphenicol palmitate suspension equivalent to 50 mg. of chloramphenicol per Kg. of body weight.

As you will notice in figure 1 in the older age group, maximum blood levels were found at the two hour sampling period, indicating rapid intestinal absorption. These are similar to the adult levels found in crystalline material. These levels drop off rapidly requiring about four hours to reach half the maximum level. In the newborn group, variations were noted in the 1 to 2 day old infant. Maximum blood levels were found at the 12 hour period, indicating a slower rate of absorption. Peak levels were lower. Reduced rate of absorption was probably due to slower hydrolysis in the intestinal tract, although a lower rate of absorption has not been ruled out as a contributing factor. The blood levels in the newborn group were greatly prolonged, with an apparent half life of 28 hours.

Chloramphenicol succinate is a highly water-soluble ester of chloramphenicol and is designed for parenteral use. It is rapidly absorbed from the site of injection and is distributed throughout the body in the extracellular water. Hydrolysis occurs in the tissues with the liberation of free chloramphenicol. However, in studies with this product form, we utilized three different age groups in which to study the blood levels. The first group of 3 children were 4 to 5 years of age and they were given a single subcutaneous dose—injections of succinate in doses equivalent to 50 mg./Kg. Another group, 3 infants 10 to 16 days of age, was given intramuscular doses equivalent to 25 mg./Kg., and the third, 5 newborn infants 1 to 2 days of age, was given a single intramuscular dose equivalent to 50 mg./Kg.

In all cases, high blood levels were attained rapidly (fig. 2), the half life was about 26 hours in the newborn group as compared with 10 hours in the 10 to 16 day group and only four hours in the older age group. Prolonged half life in the youngest age group coupled with rapid attainment of blood levels indicates that the elimination of nitro compounds is slow.

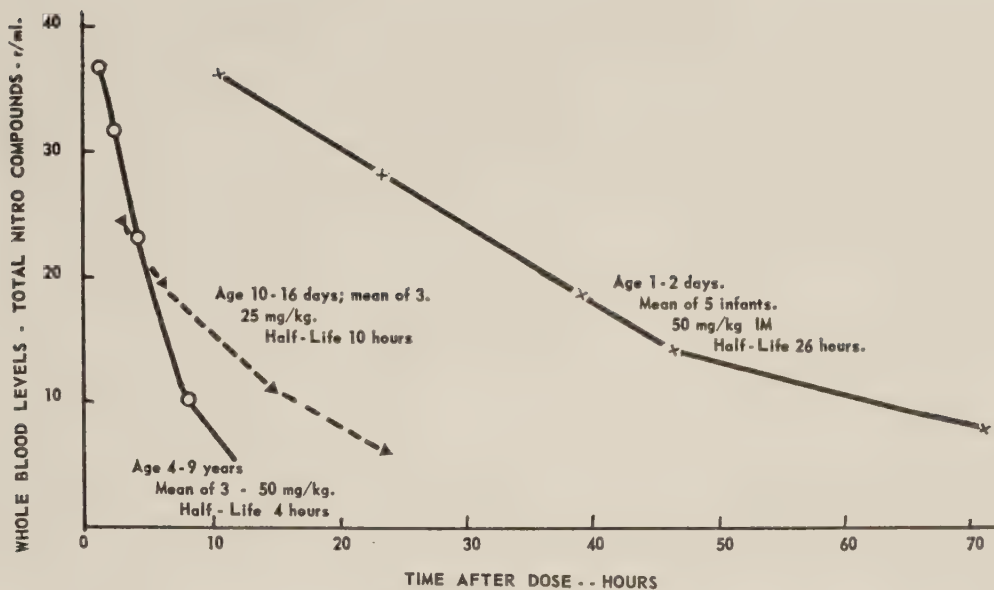


FIG. 2. Results of giving a single dose intramuscularly, 25 and 50 mg./Kg., of chloramphenicol acid succinate.

Micronized chloramphenicol is an aqueous suspension of micronized chloramphenicol crystals in the micron range, designed for intramuscular administration. Being only slightly soluble in water, some degree of repository effect is anticipated. Where renal function is deficient, even greater prolongation in blood levels would result. As this occurs in infants, progressively higher blood levels might be suspected following repeated injections of this preparation. A single intramuscular dose of 50 mg./Kg. was administered to each of 9 newborn infants one to two days old, blood specimens being drawn at intervals for four days. Maximum blood levels of 38 $\mu\text{g./ml.}$ were found at the 23 hour sampling period with a slow decline thereafter (fig. 3). The rate of fall in blood levels was not constant, apparently being very slow at first but increasing in the later time periods. This may be due to the continued absorption of greater quantities of the drugs in the earlier time period with less being absorbed in the later periods. Possible increases

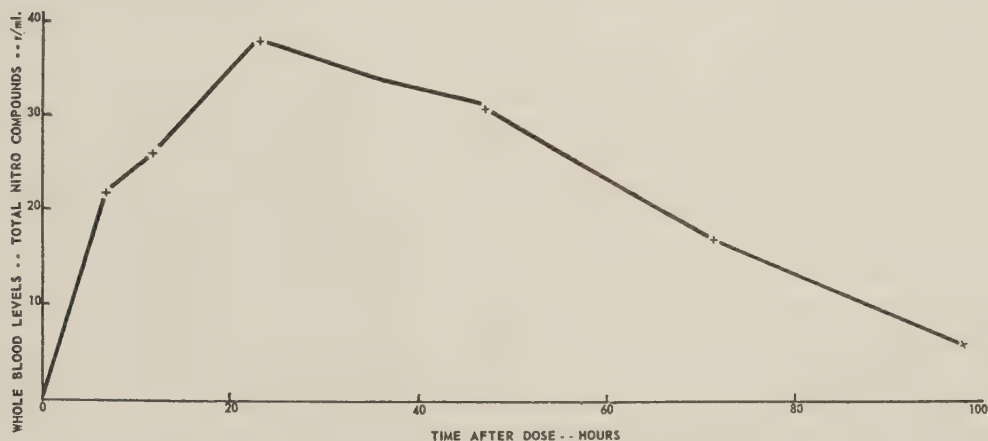


FIG. 3. Mean of 9 infants, 1 to 2 days of age, given a single intramuscular dose, 50 mg./Kg., of chloramphenicol.

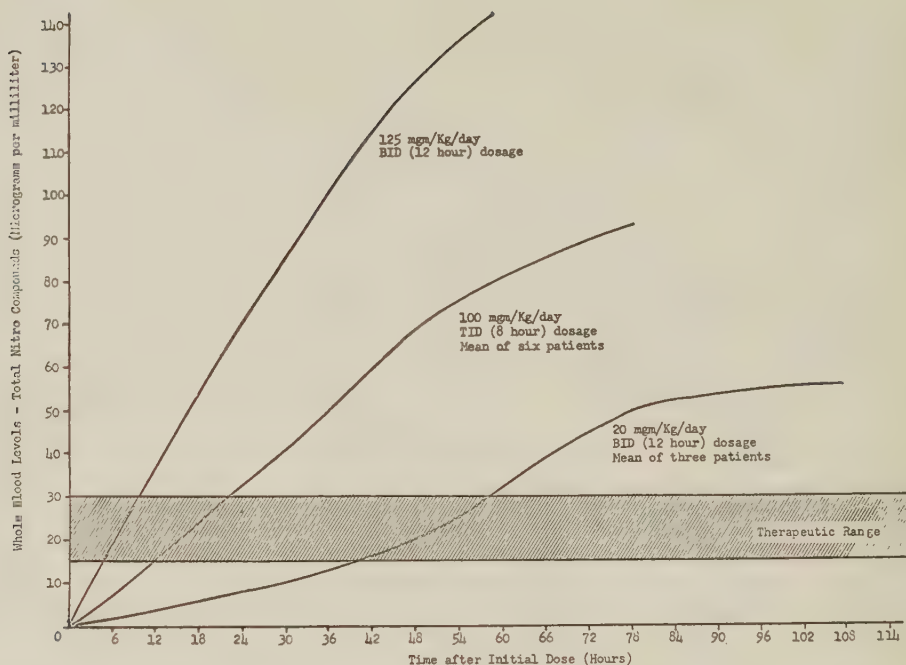


FIG. 4. Total nitro compounds in whole blood of premature and newborn infants in neonatal period. Intramuscular chloramphenicol was used. In premature and newborn infants the microbioassay approximates 50 to 75 per cent of the colorimetric determination.

in functional capacity of the hepatic and renal systems may also be involved as the infant matures.

Repeated doses of chloramphenicol have been recommended at 8 to 12 hour intervals for the maintenance of adequate therapeutic levels. The effect of such multiple dose schedules on the blood levels of newborn infants was investigated at different daily dose levels. Infants ranging in weight from 1.4 to 3.9 Kg. were given intramuscular doses of this preparation ranging from 20 to 125 mg./Kg. each day using equally divided doses every 8 to 12 hours (fig. 4). With doses of about 100 mg./Kg. daily, blood levels reached values of 100 μ g./ml. By the third or fourth day, which is the time period at which early symptoms of the gray syndrome may first appear, we find these high levels being attained. Even with low doses of 20 mg./Kg. daily, the levels may exceed 50 μ g./ml. by the fourth day. However, no side reactions were found at this lower dose schedule.

In summary, chloramphenicol blood levels in the newborn infant differ in several important respects from those in the older child. Levels are maintained for a longer period. The concentration of this or any drug may be affected by two main processes, the rate of absorption and the rate of elimination. These may include such factors as the rate of hydrolysis of esters, protein binding, diffusion in the tissues, detoxification processes and hepatic as well as renal excretion. The previously known metabolites of chloramphenicol have shown low toxicity in rats and in adult human anuric patients. A new metabolite, glucuronide glycolic acid amide aminodiol hydrolysis product, has been checked and likewise shows low toxicity. Side reactions accompanying the administration of large doses must be due to the free drug itself rather than its metabolic products. The amount of chloramphenicol excreted in the urine of newborn infants appears to be in the normal range, 5 to 10 per cent of the dose. The excretion of glucuronide is below normal, that is 50 per cent of the dose. High prolonged levels of free chloramphenicol in newborn

infants appear to be due to deficiencies in the conjugating system as well as to poor renal function.

Newborn animals present challenging problems in drug dosage, especially because of deficiencies in the various enzyme systems. They appear to be more sensitive generally to the effects of drugs. The doses must be studied further to avoid toxic reaction. Toxic effects of chloramphenicol have been reported to be greater in newborn than adult rats. Progesterone normally conjugated with glucuronic acid is extremely toxic to newborn mice. Conjugation of N-acetyl *p*-aminophenol with glucuronic acid is retarded in newborn infants. A water soluble vitamin K analogue has been shown to produce hemolytic anemia and kernicterus in the newborn. Newborn infants receiving certain sulfonamides also showed an increased incidence of kernicterus. The mechanism for acetylation of sulfonamides is impaired in the neonatal period. From this, it is clear that the greater toxicity of drugs to the newborn is not limited to chloramphenicol alone.

Variations in the metabolic disposition of chloramphenicol in the newborn infant have been discussed briefly. Reasons for these differences have been clarified in the light of the current knowledge concerning the development of hepatic and renal function in the newborn. Variations between different preparations have been discussed.

It is hoped that this discussion will stimulate thought, experimentation, study, and development of new therapeutic approaches to therapy in the newborn with any drug. Only by this furtherance of investigation may the physician proceed with the therapeutic regimen in the newborn with safety, efficacy, and wisdom.

Dr. Ross (Moderator): Dr. Weiss, I would like to ask you one question, if I may. From your data, would you say that the cut-off period occurs, when can you give chloramphenicol in the usually prescribed dose of 100 mg./Kg./day? In other words, your data would indicate you are considering mostly infants in the 1 to 2 day old age group, so when do you think you could use chloramphenicol with impunity without the possibility of producing the gray syndrome?

Dr. Weiss: We must realize that there are possibly disease entities in combination that might present much the same problem as the premature or newborn era. In other words, renal or hepatic deficiencies. These, the physician must take into his consideration with any treatment with this drug, as would be true with any drug metabolized in this manner. Excluding this, we have not been able to pin down the exact time, whether it be 18, 30, or 50 days in which the majority, that is 90 per cent or so, of the infants achieve a mature response in so far as levels are concerned.

I would say that when clinical findings warrant, recommended doses may be exceeded as a broad range of safety exists. In order safely to maintain increased levels the micro-technique should be employed. In the very young infant, when prolonged therapy or usual recommendations are exceeded, one should be guided by blood level determinations.

Dr. Ross (Moderator): What would be your optimal dose then in that age group?

Dr. Weiss: Optimal dose depends upon what is being treated, Dr. Ross. In an apparently full-term infant with no complications with a moderate infection in which we should like to have a blood level of 25 μ g., we can use 50 μ g./Kg./day with perfect safety and achieve these levels during the greater portion of the day.

Dr. Ross (Moderator): Now I would like to have our second panelist, Dr. High, discuss a problem that is extremely important in the pediatric age group, namely, chronic bronchopulmonary infection. Dr. High.

Dr. High: The comments I have to make come from experience in hospital practice and not in office or in home practice. Further, these observations are made from our experience in one institution over a period of time. Therefore, they may not necessarily be appropriate for problems in your immediate area, but I think the general type of patient that we refer to here is well known to everyone.

The extent of the problem of chronic bronchial pulmonary disease in children, as we see it in Philadelphia, is relatively low in the general population, but it does constitute a very common group of hospitalized patients. I would say that we almost always have more than 15 per cent of our beds occupied by patients who have a variety of the diseases that produce chronic bronchial pulmonary disease. Now these can be grouped into four major headings. Our most common problem of this nature relates to those infants and children who have cystic fibrosis of the pancreas.

Next we would have other specific chronic disease problems in which infection is a part, although it may not be the basic part. There are a wide variety of different diagnoses to be considered in this group, including fungal infections, parasitic infections, the problems related to bronchial asthma with superimposed infection, chronic sinus disease, and the like. Then we have chronic nonspecific factors in which infection is probably of only secondary importance; aspiration of a child with neurological damage is a common problem. Bronchopulmonary disease secondary to severe congenital heart disease is extremely troublesome. This is particularly common in those infants with cyanotic heart disease who are in chronic low-grade failure. Bronchiectasis, which numerically are not particularly important is also in this category.

The obvious problem is to try to establish diagnosis with all the appropriate things that one must do. One of the things that we feel has considerable importance is the difference in the bacteriologic studies depending upon whether the samples are taken from the nasopharynx or from bronchial aspirate.

It would appear relatively important to pay particular attention to the bronchial isolates because they probably represent the significant bacterial pathogens in these patients. Staphylococci are isolated singly or in combination in very large numbers from more than 85 per cent of patients who have had manifestations of cystic fibrosis for any period. The other gram-negative bacilli are also very prevalent, especially coliforms and *Proteus*. Some of these are relatively infrequent in the patients without cystic fibrosis. The gram-negative bacteria, instead of constituting approximately 75 per cent, constitute only 25 per cent. Pneumococci are isolated in a far greater number and so is *Hemophilus influenza*. There are some geographic variations relative to the isolation of *Hemophilus* in cystic patients in this country but at least in our clinic these are found relatively infrequently.

The staphylococci are a problem from the standpoint of eradication, and they tend to be resistant in patients who have chronic manifestations of cystic fibrosis of the pancreas. These organisms show resistance to a variety of antibiotics. There are staphylococci that are inhibited by concentrations less than 15 µg./ml. of the various antibiotics, but the great bulk of these are refractory to almost all antibiotics. Perhaps the fact that few are novobiocin-resistant is a reflection of the fact that we have withdrawn the use of novobiocin to a large extent in our institution in the past two years.

I do not think there is any comment to make about the treatment of the patients with chronic lesions secondary to tuberculosis. This disease is probably one of the easiest to treat. Of recent interest has been the introduction of the use of corticosteroids on a trial basis in patients with bronchial obstruction or pleural effusion. These results, at the moment, have no real comparison for evaluation but the Trudeau Society is undertaking this study to try to establish their value. Patients with the remaining types have problems relative to eradicating chronic infection; this may be extremely difficult.

Common to the groups with cystic fibrosis and the other chronic specific or non-specific diseases would be the use of systemic antimicrobial therapy, either on a continuous, an intermittent, or prophylactic basis. This cannot be generalized upon because the severity of the patient's disease at the moment will dictate what must be done. Some of the severe cases with cystic fibrosis require continuous, or almost continuous, therapeutic blood levels. Others, with relatively minor component of pulmonary disease, may require treatment only if they have an intercurrent infection.

Aerosols are extremely difficult to evaluate. We have attempted to do this but there seems to be no practical objective measure of the value of aerosol therapy. Some patients, who are older, claim to feel better and seem to bring more sputum up when they have had such treatment. Aerosol enzymes have, as far as we can tell, no way of practically assessing their value. Older children in general tend to state that their cough is looser following the administration of these agents. For all of the patients, other than the patients with tuberculosis, bronchoscopy with aspiration and direct mechanical removal of large amounts of purulent material may be beneficial. For all of these patients, the tubercular, the cystics, and the others, we feel that consideration should be given to the prevention of viral infections as far as is practical because many patients have a flare-up of staphylococcal infections, in particular following immediately after viral infections. This, of course, can be done for only a few things—modification of measles, influenza vaccine, and possibly adenovirus vaccine. The outlook in these patients depends on the type—for tuberculosis it is really remarkably good. We have not had a death in our institutions since 1950 from patients with pulmonary tuberculosis. All of the deaths in tuberculosis have occurred in infants and children with tuberculous meningitis.

Patients with cystic fibrosis of the pancreas represent a group in which the outcome is almost impossible to evaluate. However it is becoming apparent that more children can be carried through infancy and childhood with relatively good pulmonary function remaining and that some of these patients have now reached their late teens and early twenties. Admittedly most of these are in rather difficult condition in terms of pulmonary function. Our oldest patients are 17 and 20, the former is a serious pulmonary cripple and the latter has very little disability.

For the other chronic nonspecific or specific processes the ultimate outcome, of course, depends to a large degree on the cause. Some of those in whom we never find a cause seem to recover for reasons we cannot explain.

Dr. Ross (Moderator): Thank you, Dr. High. Now I am going to turn the rest of this panel over to Dr. Sprunt.

Dr. Sprunt (Moderator): The geriatric portion of this panel has arranged three short talks, one on the use of antibiotics in acute infections in the aged, another on the use in chronic infections, and lastly on the prophylactic use of antibiotics in the aged. We shall leave some time for questions at the end. The first paper will be given by Dr. Goodman.

Dr. Goodman: It is both a privilege and an honor for me to participate in this panel on the chemotherapy of acute and chronic pediatric and geriatric infections. At the outset, I must admit that personally I feel more qualified to speak on the geriatric aspects of the subject than on the chemotherapy of infections. My basic discipline is not in infectious diseases. However, recently at the Daniel Drake Memorial Hospital and Hamilton County Home, an institution devoted to the care of the long-term patient, we have become interested in the evaluation of certain antibiotics in the treatment of both

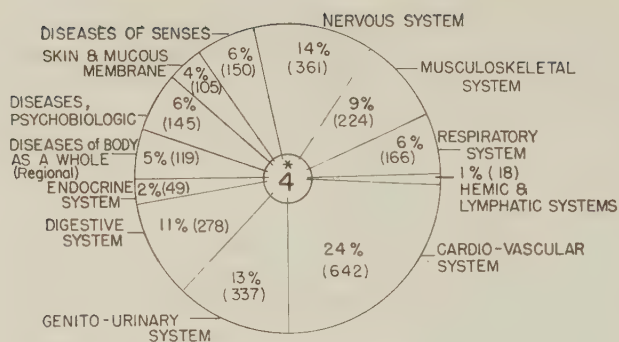


FIG. 5. Incidence of diseases in geriatric patients at the Daniel Drake Memorial Hospital as compiled by systems, December 31, 1953. Total of 2594 diseases in 597 patients. 4* = average of 4 separate disease entities per patient.

acute and chronic infections. A summary of the clinical experiences, representing the work of a number of my associates on the staff of this institution, will be reported this afternoon. These projects were undertaken because of the paucity of reports dealing with this subject and in order to find an effective antimicrobial agent that could be tolerated by the aged. Most reports on the treatment of acute infections are of otherwise healthy individuals. The majority of the patients in our institution suffer from multiple chronic diseases: many are hospitalized for long-term active medical treatment, others require predominantly nursing care, and a smaller number of the aged need custodial supervision. These patients live in an institutional environment and hence are apt to be infected by organisms that are more resistant to antibiotic therapy. They are often debilitated by severe coexisting diseases involving many systems.

On the average, each patient has an involvement of four major organ systems. Since added acute infection poses a serious threat, a particular medication could be relied upon only if response were prompt.

In figure 5 can be seen the high incidence of multiple diseases in the patients of our institution. At least four major organ systems were involved with disease or disability. The figures are taken from the inpatient register of 1953, when the daily census was about 600 patients; today it is nearer 800, but the type of patient admitted is still the same. This figure emphasizes the complexity and the chronicity, as well as the multiplicity, of disease that exist in the patients that were under observation and treatment.

In figure 6 the analysis of diseases present in the patients were taken from an analysis of 266 deaths. Most of the patients suffered from at least five major causes of death, at least five major systems were involved in this instance. Notice at the bottom of the section, that the increase in respiratory infections is up to 17 per cent and in figure 5 it was 6 per cent.

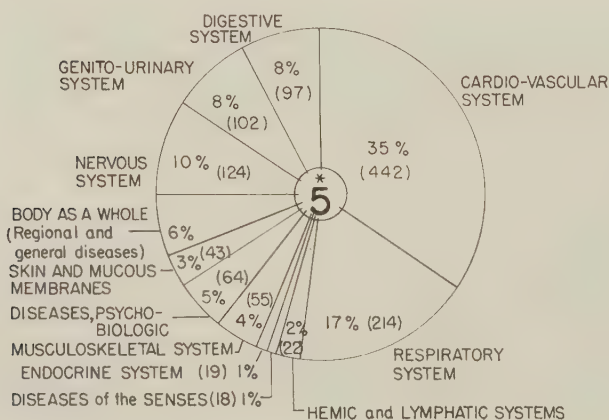


FIG. 6. Deaths from principal and underlying causes in geriatric patients at the Daniel Drake Memorial Hospital. There were 1272 causes of death in 266 deaths. 5* = average of 5 separate disease entities per patient.

TABLE I

Results of Treatment of Acute Bronchopulmonary Infections with Penicillin V

Sputum culture	No. patients	Results*		
		Good	Fair	Poor
<i>D. pneumoniae</i>	1	1		
<i>Staph. aureus</i>	5	3	2	
Mixed flora	5	3	1	1
<i>H. influenzae</i>	1			1
No culture	11	6	2	3
Total	23	13	5	5

* Good = prompt clinical and laboratory response; fair = delayed clinical and laboratory response; poor = no response.

These investigations were done with both phenoxymethyl penicillin and also erythromycin propionate. First, let us consider the discussion of our investigations with penicillin V in the treatment of acute infections. These studies were done in collaboration with Drs. Nell K. Levy, Leonard Burgin and Stanley D. Simon of our staff.

It has been demonstrated that adequate penicillin blood levels are obtained when penicillin V is given orally and it is an effective agent in the treatment of certain infections. As has been said, its value in geriatrics in chronically ill patients to our knowledge has not been previously reported.

Accordingly, 63 patients with a variety of infections were given penicillin V. The patients were evaluated clinically and appropriate roentgenograms and laboratory studies were made whenever possible. Variation in dosage was utilized in accordance with the severity of the infection but the range was from 250 to 375 mg. four times daily.

Of these patients, 28 were grouped together because in these penicillin was given either together with another antibiotic or because it was given prophylactically or for indeterminate situations. This group will not be discussed this afternoon.

Thirty-five patients with acute infections of the pulmonary system or infections of the soft tissue or bone were treated with penicillin V alone and these results will now be discussed. Table I lists data for 23 of the patients with acute bronchopulmonary infection, 11 had pneumonia and 12 had acute bronchitis. The average age group of these patients was 67 years and the length of therapy varied from 6 to 35 days.

Table II lists soft-tissue infections and the results of therapy with penicillin V. In patients with cellulitis and diffuse infections of the soft tissues, results were good in 5

TABLE II

Results of Treatment of Soft-tissue Infections with Penicillin V

Condition treated	Number patients	Good	Poor
Cellulitis	2	2	
Erysipeloid eruption	1	1	
Dental abscess	1	1	
Abscesses	2	1	1
Chronic leg ulcer	1		1
Paronychia	2		2
Osteomyelitis	1		1
Infected finger	1		1
Infected traumatic bursitis	1		1
Total	12	5	7

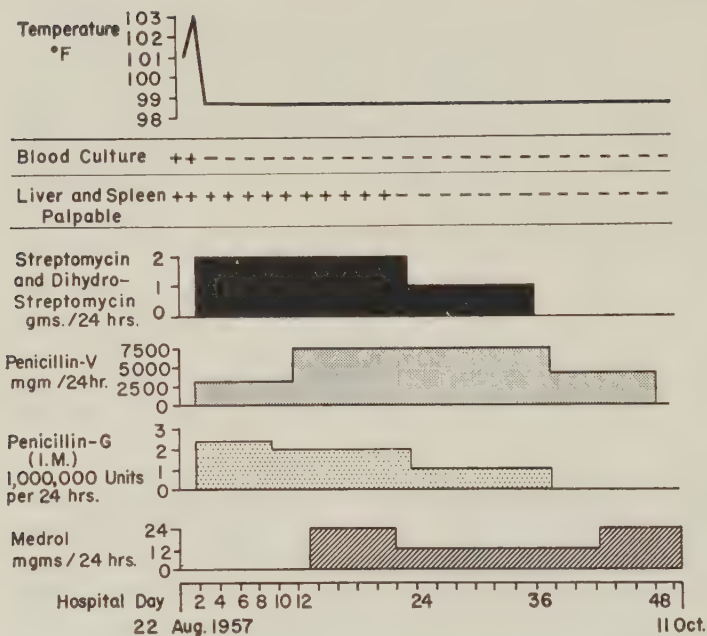


FIG. 7. Enterococcal endocarditis in a 95 year old white patient successfully treated with oral penicillin.

instances. In the other 7 instances, the result was poor. Some of these patients later responded to other agents or to incision and drainage. Acute resistant staphylococci were found in several.

In figure 7 is depicted a graphic illustration of a patient who, at 95 years of age, suffered from an enterococcal endocarditis. Sometimes a graphic illustration of one patient gives one a better idea of the effectiveness of an agent than a mere recitation of statistical data. This patient had a high fever, he was given chiefly penicillin V orally, since he refused injections. We did give streptomycin by injection and some penicillin G at the same time that streptomycin was given. According to Hunter, at least 10 to 15 million units of penicillin is necessary in the treatment of this disease and here you can see that 80 per cent of the penicillin given to this individual was in the form of penicillin V. He was treated for eight weeks in all.

I would now like to consider our experiences with erythromycin propionate. This study was done in collaboration with Drs. Gordon Mindrum, Paul Geiss, and Leonard Burgin. This is a preliminary report of investigations still in progress. Fifty-six selected patients who developed acute infections while under treatment for a variety of multiple chronic diseases were treated with erythromycin propionate. The purpose of this study was to determine the effectiveness of this agent in a variety of infections involving the pulmonary system, the upper respiratory system, soft tissues, and bones. Erythromycin

TABLE III
Results of Treatment of Pneumonia with Erythromycin Propionate

Sputum culture	Number	Good	Poor	Indeterminate
<i>D. pneumoniae</i>	6	5	1	
<i>Staph. aureus</i> (coagulase positive)	3	2	1	
<i>Staph. aureus</i> (coagulase negative)	1	1		
Normal throat flora	10	9		1
No culture obtained	11	10		1
Total	31	27	2	2

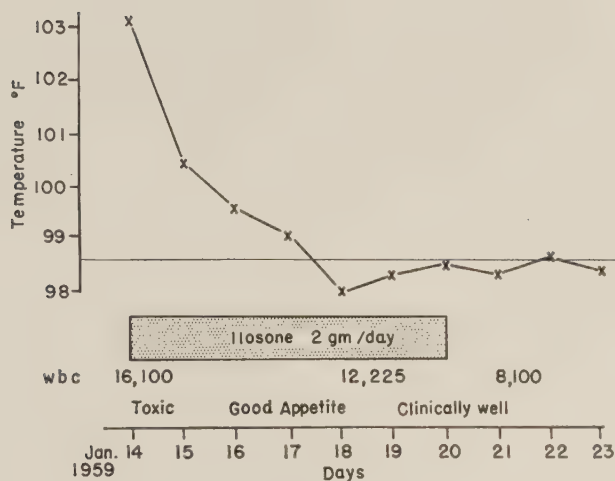


FIG. 8. Pneumococcal pneumonia in a patient who was treated with erythromycin propionate.

propionate produces predictable high blood concentrations within 30 minutes after ingestion and the peak blood levels are four times as high and persist for several hours longer than with an equivalent amount of erythromycin. This drug is reputed to be free from toxic, allergic or other side reactions. The antibacterial spectrum is identical with that of the parent compound, erythromycin. All of the patients had already been studied completely for the underlying conditions and were re-evaluated when they developed a superimposed infection. The type of patient treated was much the same as that encountered in the previous study. The dosage of the medication was 0.25 to 0.5 Gm. four times daily. Complete clinical studies, as well as laboratory tests were done when necessary. Cultures were made in all but 13 patients. The results obtained in this series of patients are tabulated. Thirty-one patients were treated for pneumonia; in 27 the response was good, and in 2 poor and in 2 the result was indeterminate (table III). The average age was 73 years, the range in age was from 53 to 92 years. After following the pediatricians, we might point out that we are speaking in years, not days. In 6 patients, pneumonia was superimposed on pre-existing chronic bronchopulmonary disease;

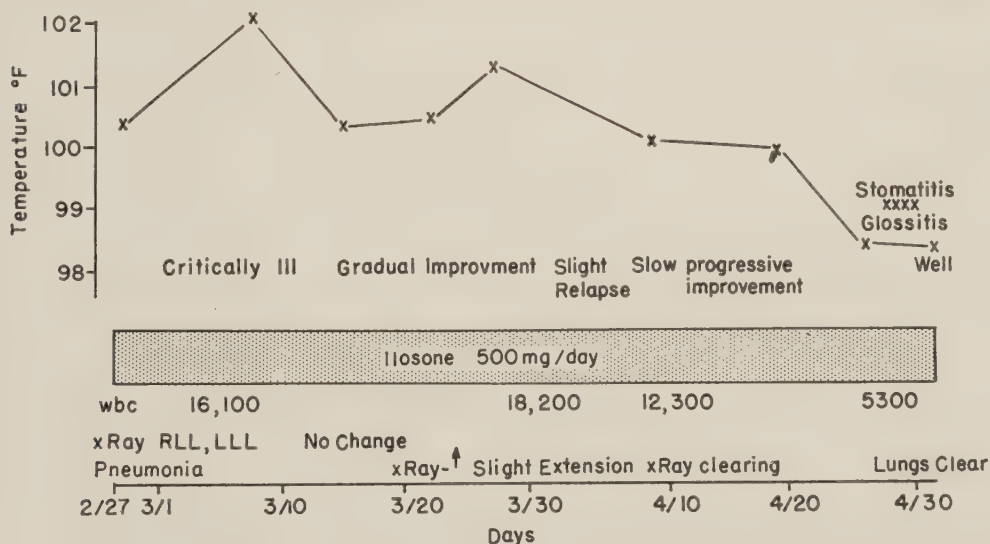


FIG. 9. Results of treatment with erythromycin propionate in a patient with staphylococcal pneumonia.

TABLE IV

Results of Treatment of Soft-tissue Infections with Erythromycin Propionate

Condition treated	Predominant organism	Number patients	Good	Poor
Abscess and carbuncle	<i>Staph. aureus</i> (coagulase positive)	5	5*	
Cellulitis	<i>Staph. aureus</i> (coagulase positive)	3	3	
	<i>Staph. aureus</i> (coagulase positive) plus <i>Proteus</i>	1	1	
Pyodermia with cellulitis	<i>Staph. aureus</i> (coagulase positive)	4	4	
Total		13	13	

* Required incision and drainage in addition to antibiotic.

in 5 patients previous therapy with other antibiotics had failed; a number of patients suffered from chronic brain disease and aspiration pneumonia was present. In 2, no fever was reported. The average duration of treatment was 15 days, the range was from 6 to 62 days. The sputum cultures revealed the presence of pneumococcus, *Staphylococcus*, and normal throat flora; in 11 we could obtain no culture at all. Of the 2 patients who had a poor result, 1 was a staphylococcal pneumonia and the other was a pneumococcal pneumonia. Of the 2 patients with an indeterminate result, 1 developed a cerebrovascular accident, while improving from the pneumonia, and the other patient developed a hemorrhagic cystitis and another drug had to be used in the treatment. Perhaps both should also be included among the poor results.

Figure 8 shows a representative example of the response to therapy of a patient with pneumococcal pneumonia. Thirteen others had a similar response, and in some we could find no specific organism. You will notice that this 88 year old woman responded promptly to erythromycin therapy. The onset of illness was sudden, with fever, chills, high white count, and bilateral pneumonia proved by roentgenograms. The response was quite

TABLE V

Results of Treatment of Miscellaneous Infections with Erythromycin Propionate

Condition treated	Predominant organisms	Number patients	Good	Poor	Indeterminate
Osteomyelitis	<i>Staph. aureus</i> (coagulase positive)	2	2		
Purulent arthritis	<i>Staph. aureus</i> (coagulase positive)	1	1		
Otitis media	<i>Staph. aureus</i> (coagulase positive)	1	1		
Acute pharyngitis and bronchitis	α -Hemolytic <i>Streptococcus</i>	2	2		
	No culture	2	2		
Acute pyelonephritis	<i>Staph. aureus</i> and <i>E. coli</i>	1		1	
Septicemia	Unidentified gram-negative rod	1		1	
Pulmonary tuberculosis.	Acid fast bacillus	1			1
Granuloma of undetermined etiology		1			1
Total		12	8	2	2

TABLE VI

Side Reactions in a Series of 56 Patients Treated with Erythromycin Propionate

Complications	Number patients	Remarks
Nausea and vomiting	4	Treatment was continued
Stomatitis		
Moniliasis	1	Cleared with gentian violet
<i>Staph. aureus</i> coagulase positive	1	Responded when primary condition improved
Tarry stool	1	Source not found
<i>Staphylococcus</i> resistance	2	After 15 days treatment

dramatic in this patient. After seven days the pneumonia cleared and she was clinically improved. The chronic brain syndrome, of course, was unchanged.

Figure 9 represents a graphic illustration of a patient who had a staphylococcal pneumonia. You can see that this illness lasted for 62 days, and the patient's illness was much more severe. The response to therapy, although slow and gradual, was ultimately effective. There was not only clinical improvement of the patient but also confirmatory laboratory data. Two complications occurred in the course of therapy. A tarry stool was passed on about the forty-fifth day of therapy, and we never found the source of bleeding. Smith has reported a similar complication, actually a fatal complication, in one of his patients who was on therapy and he was also unable to find the source of bleeding. Our patient also developed glossitis and stomatitis in which monilia was grown, which responded to local gentian violet therapy.

In table IV we summarize our experience in the treatment of soft-tissue infections. The predominant organism was the *Staphylococcus* and of the 13 patients treated for cellulitis, abscess, and carbuncle, all responded very well. One patient, a diabetic woman, had a large abscess of the axilla in the chest area, which responded very promptly. Another diabetic with a large carbuncle on the neck, responded perfectly well. Incision and drainage was necessary in both of these instances, as well as in three other patients.

In table V we summarize our experience in the treatment of a miscellaneous group of infections. Osteomyelitis responded perfectly well, as did bronchitis, purulent arthritis and streptococcal pharyngitis. However, of the 2 patients whom we have recorded as indeterminate, in one, after a month's therapy, the sputum cultures came back positive for tuberculosis; the other patient had an apical granuloma that was proved by thoracotomy. We did obtain a poor result to treatment in a patient with an associated acute nephritis in one patient with a staphylococcal infection, and in one with an unidentified gram-negative bacillus septicemia.

In table VI are listed the side reactions that we experienced with the use of erythromycin propionate in our series of aged individuals with acute infection who were also suffering from a multiplicity of chronic diseases. In 4 instances the symptoms of nausea and vomiting were transient and we did not have to stop the use of the drug.

In closing then, penicillin V was used in the treatment of a variety of infections in 63 geriatric patients alone or together with other antibiotics and was found to be well tolerated. In 35 acute infections, it was used alone and found to be very effective. A 95 year old man with enterococcal endocarditis was treated successfully with penicillin V and streptomycin and a small amount of penicillin G by injection. Lastly, erythromycin propionate was used in a series of 56 geriatric patients with a variety of infections and was found to be well tolerated and very effective, particularly in staphylococcal diseases. The results obtained with this drug appear to be superior to those obtained with penicillin V in a similar group of patients.

Dr. Sprunt (Moderator): Thank you. Now we will hear the next speaker, who will talk on the use of antibiotics in chronic diseases in the elderly. Dr. Lepper.

Dr. Lepper: The report I am going to give concerns a double blind study that we have been doing in people with chronic bronchitis and/or bronchiectasis. More than 75 per cent of the patients had bronchiectasis proved by roentgenogram, and the majority were older than 50 years of age. In picking the drugs to use we were quite interested in the problem of the relative specificity of *Hemophilus influenzae* as a causative organism in chronic pulmonary disease in adults. For this reason, we chose to study drugs that we hoped would differentiate the effect against many organisms including *Hemophilus* from an effect against many organisms but which left *Hemophilus* relatively untouched and from an effect against only those things that cause acute pulmonary disease in adults frequently, such as the pneumococcus. Therefore, the therapeutic groups we have studied have been first of all a placebo, with 23 patients; second, penicillin, 1,600,000 units by mouth a day in 24 patients; third, an oleandomycin-penicillin combination, 800,000 units of penicillin and 1.5 grams of oleandomycin a day; and fourth, tetracycline, 2 Gm./day.

Now, as I say, these were chosen in the hope of getting some selective effect on the flora and still have several groups in which a wide coverage would be obtained. I will not give the complete results today. We have been interested in studying many parameters, such as relatively complete pulmonary function testing including measurements of ventilatory capacity, mixing of gases with the nitrogen washout, and diffusion and venous admixtures studied by the Reilly technique. These studies have not revealed much in the way of progression or regression of the disease at the end of two years.

The chemical aspects have been followed particularly with serial C reactive protein determinations, and serum protein electrophoretic patterns. One might say that in the therapeutic groups the C reactive protein levels are lower and the electrophoretic pattern definitely has improved in that the gamma globulins have tended back down toward normal and the albumin has tended to come up.

The bacteriology we will report on. We have done serial viral serology and found that there are a fair number of the acute episodes that can be accounted for, for various respiratory viruses, at least as measured by serologic changes with passage of time.

Most of the reports I will give today, however, are concerned with an evaluation of the bacteriology and evaluation of the clinical response. As I indicated, the study has been done double blind and the clinical response has been measured in terms of the number of episodes of lower respiratory infections. These were defined by a worsening of cough, change in color and/or the amount of sputum, with or without fever. We have analyzed those with fever and those without fever separately. Similarly, we have defined as upper respiratory illnesses those in which the symptomatology of the lower respiratory tract has been relatively unchanged but the person definitely has had increased amount of nasal secretion, sore throat, and the usual symptoms of upper respiratory infections, these, too, with or without fever.

These are the main clinical parameters that we have tabulated for you today. The amount of sputum has been measured, the change of color of sputum, the number of times the patients have had to be hospitalized, the number of times that the patients have had to receive antibiotics other than the basic drug regimen have also been analyzed and the results support the findings we are reporting. When the patient has needed other antibiotics, they have usually been hospitalized. Although they may have gotten the same drug in the hospital, it was usually given intravenously. It was not necessary to break the code to handle the respiratory illnesses.

The episodes of the lower respiratory infections per year; the placebo group, 2.7; tetra-

cycline, 1.4; penicillin, 1.8; and oleandomycin-penicillin, 1.4. Both the tetracycline and the oleandomycin-penicillin groups are significantly different from the placebo group but the penicillin result is not significantly different from either of these drug groups. The two effective drug groups were not different. Penicillin is only a borderline significantly lower than placebo.

Similarly the episodes of lower respiratory infection with fever, the tetracycline was the most antipyretic drug, and one wonders about the specificity of this effect, since the number of episodes were not decreased below oleandomycin-penicillin but one can see here that there was a lowering of the number of febrile episodes per year with tetracycline and oleandomycin-penicillin made less difference.

Similarly, we have analyzed the days of lower respiratory illness per year: the placebo group averaged 21 days per year; tetracycline, 13; oleandomycin, 9; and penicillin, 20. In this regard, the oleandomycin-penicillin group looks a little better than the tetracycline group, in that the oleandomycin-penicillin results were significantly better than those with placebo or penicillin.

In the data for the days of lower respiratory infection with fever, the fever was reduced considerably but because of the small numbers there are no significant differences when tested by the statistical method. However, these results were about the 10 per cent level.

We have analyzed the upper respiratory infections more or less as a control on these results. These are episodes of upper respiratory infections and there were no really significant differences, although interestingly enough there was a slight reduction with oleandomycin-penicillin of upper respiratory episodes.

In the upper respiratory infections with fever the item of borderline significance is that even though the episodes were not decreased, there was a slight reduction of febrile episodes with tetracycline even in the upper respiratory infection. Again this raises the question of how much antipyretic effect the tetracyclines might have. One recalls that this is an old debate in the literature, there being animal studies on both sides of this problem of antipyretic effect.

As to days of the upper respiratory illness, all the drugs had fewer days as if they may have been cutting down the length of the respiratory illness somewhat in these patients, but only the oleandomycin-penicillin versus placebo figure is significant.

In upper respiratory infection with fever, the number of febrile days is variable enough so that there were no real significant differences although there was a slight reduction again in the tetracycline group that gives us the same pause that we had before.

The results of the bacteriology showed that there were significantly fewer positives, only 13 per cent of all cultures after treatment was started, in the tetracycline group. I may say in contrast to the material Dr. High presented for children, *H. influenzae* can be found in about 90 per cent of these patients at some time by using the techniques published by Mulder and his group in Holland, where one washes the flecks of sputum to get rid of the upper respiratory organisms. When this is done a very high yield of *Hemophilus* organisms is obtained. These are not *Hemophilus* group B, these are non-typable, which are determined by the strain and cultural characteristics and growth requirements.

Another point to emphasize is that the *Staphylococcus* has been infrequent in this group and indeed this was a surprise to us. We, too, have been impressed in the few fibrocystic children we have seen in consultation of the importance of the *Staphylococcus* so that the relative infrequency of *Staphylococcus* here, even in the placebo group, is noteworthy.

The pneumococcus has been reduced significantly in all the drug groups, as one might suspect, and in spite of the fact, of course, that the pneumococci have been held in check with penicillin, there has not been a favorable therapeutic result in the group. One can see that there was some increase in the gram-negative rods in the tetracycline

group and in the penicillin group and that the beta-hemolytic *Streptococcus* has not been a particular problem when one gets away from the upper respiratory tract in these people. *Pseudomonas* was also increased. In spite of these increases, however, there is no good evidence that they contributed to symptomatology. The yeast has been so variable that we have not found them to be of any significance. As one can see, they have not been increased in frequency over the placebo by the use of the drugs.

We have been impressed by the relative tolerance of the patients to these drug regimens. As I have indicated, the original patients are now in the third year of continuous antibiotic medication and in spite of this we have not had to stop anyone for toxicity. We have had to reduce the dose in about 5 of the tetracycline patients for varying periods of time because of gastrointestinal upsets but ordinarily after being on the reduced dose for a short period of time the dose can be raised again. This has been quite a surprise to us and I believe it will come as a surprise to some of the people that have used tetracycline and oleandomycin-penicillin combinations before.

In summary, I might say, we are not necessarily recommending that this is the best way to manage the bronchiectasis patients. One can see from the number of episodes and the number of days of illness per year that an impressive fact is that even though these people are chronically ill, they are not sick all the time and that we are measuring relatively small differences of morbidity.

I would concur with Dr. Goodman's statement that when you treat older people you treat people with a great many other diseases and I do not want to give the impression that this is a disease-free group. Most of them also had two or three other systems involved, some of them already had cor pulmonale when we started the study. However, the progression of the disease has been so slow that it has been very difficult to measure so that we were left with measuring the rates of morbidity to date and some chemical and, we hope, functional parameters of the situation. So far we have been impressed by the relatively good results that we have seen.

Dr. Sprunt (Moderator): I have been interested in prophylactic use of antibiotics in the aged now for more than five years, working first with Dr. Leon McVay and later with Dr. Clyde Flanigan. We have, in several papers, reported the good effects of antibiotics on the general health in the aged. This has been noted particularly in patients with congestive heart failure and bronchiectasis. The results just given by Dr. Lepper concurred with ours, since he also obtained good results in bronchiectasis. However, he used a much larger antibiotic dose than we did. In our experiment we first used a total of 500 mg. a day, divided into three doses and later this was reduced to 300 mg. I do not intend to review this work, for I am convinced that the prophylactic use of small amounts of antibiotics, when accompanied by adequate vitamins and minerals, is an excellent thing in the aged.

The question has been raised that the continued use of an antibiotic may be dangerous to a person, as it might increase the incidence of staphylococci in the throat and intestine, which are resistant to antibiotics.

It was therefore decided to investigate this problem. The inmates of a county home for the aged were chosen for this study. At first, two groups of approximately 50 persons were chosen. One of these groups received a pill containing a good supply of vitamins and minerals. The other group received a pill identical in appearance with the same vitamins and minerals and also chlortetracycline sufficient at three pills a day to give a dose of 500 mg. Later, three more groups were added. One of these new groups of 50 patients received vitamins and minerals, another vitamins and minerals with tetracycline (sufficient for 300 mg. a day), and a third group received a pill containing only lactose. The last groups were added as an additional control.

TABLE VII

Results of Bacteriological Cultures of Patients on Chlortetracycline and Tetracycline Prophylaxis Programs

	Staphylococcal isolates														
	Total cultures			Sensitive						Resistant					
				Throat		Stool		Total		Throat		Stool		Total	
	Throat	Stool	Total	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Vitamins and minerals	347	348	695	34	9.8	37	10.6	71	10.2	15	4.3	30	8.6	45	6.5
Vitamins and minerals plus chlortetracycline	336	333	669	35	10.4	27	8.1	62	9.3	10	3.0	30	9.0	40	6.0
Lactose	297	295	592	20	6.7	38	12.9	58	9.8	8	2.7	56	19.0	64	10.8
Vitamins and minerals	307	311	618	18	5.9	25	8.0	43	7.0	6	2.0	68	21.9	74	12.0
Vitamins and minerals plus tetracycline	260	260	520	14	5.4	25	9.6	39	7.5	11	4.2	45	21.8	56	10.8

In all of these groups neither the inmate, technician, nurse, or physician knew which patient received the antibiotics and which did not.

Stool and throat cultures were taken on each inmate at three month intervals. No significant changes between the groups were seen. There was only an occasional yeast seen and although the types of bacteria fluctuated with the time of year, there were no differences between the different groups. Also, we do not have definite statistical proof at this time, but it appeared that the number of staphylococci present has increased in the last year.

The main point of the experiment is shown, however, in table VII, which shows conclusively that there were no significant differences between the inmates receiving the antibiotics and those who did not.

In conclusion, the use of small dosages of an antibiotic, when accompanied by adequate vitamins and minerals, is a safe procedure in the aged.

Now, I would like to ask Dr. Ross if he would like to say anything in closing.

Dr. Ross (Moderator): I would like to make just one closing remark regarding the bacteriological diagnosis of respiratory infections in the pediatric age group as opposed to adults. A throat culture obtained in a child may in no way be indicative of the organism producing the infection in the lower respiratory tract, i.e., in bronchitis and bronchopneumonia. In this regard the isolation of *Staphylococcus aureus* from the throat may not be regarded as the offending pathogen in the bronchi or in the lungs. Hence, a specific bacteriological diagnosis of lower respiratory disease in infants and children may not be readily made on the basis of routine bacteriological methods. The finding of large numbers of pneumococci, beta-hemolytic streptococci or *Hemophilus influenzae* in a throat culture usually is significant, on the other hand, and would suggest the causative age of bronchitis or bronchopneumonia, since these organisms are not generally resident pharyngeal flora.

Dr. Sprunt (Moderator): I might add in conclusion that my wife and I have just returned from a trip around the Eastern Mediterranean. We both took 0.3 Gm. of tetracycline a day and we did not have any bacterial disease during the trip.

This concludes the afternoon session and we wish to thank everybody who stayed to hear the panel.

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